Chapter 15
Filter Paper Adsorption and Ninhydrin Reagent as Presumptive Test for Gravesoil

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Abstract In the field of forensic science, the investigation of soil for the determination of decomposition by geophysical, biological and chemical methods, is a fast growing area. During decomposition different substances are formed as a result of breakdown and dissolution. The analysis of ninhydrin reactive nitrogen (NRN: ammonium and organic amines) is helpful in the investigation of the decomposition process, because such products may result from degradation of proteins and amino acids. During this research a new application form of the well known ninhydrin reagent was developed and applied to soil and gravesoil.

Soil samples were collected in the vicinity of a decomposing pig (*Sus domesticus*), after approximately 2 months. The samples were supplied by the University of Central Lancashire and transported to the laboratory on dry ice. In previously published work, NRN is extracted from soil using an aqueous extraction, whereof the liquid is used for a modified ninhydrin reaction. During this study, the samples were moisturised, and subsequently adsorbed to a filter paper. The paper was treated with ninhydrin reagent.

The soil samples taken in the direct vicinity of the cadaver, gave positive results in the form of a deep purple colour. Soil samples taken in the same area resulted in a slight purple colour change, indicating low concentrations of NRN as well.

This ninhydrin reagent application is not validated yet and may need some further development. Nevertheless, this rapid test may complement the analytical methods described in the literature and contribute, as a presumptive test, to the field of forensic taphonomic research.

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15.1 Introduction

In fatal crimes the perpetrator often tries to hide the corpse to avoid detection (Pringle and Jervis 2010; Pringle et al. 2010). The most common approach for hiding the corpse is a quickly dug grave, which is normally dug into or nearby the topsoil surface. These burials are defined as clandestine graves which will give a confounding effect on the used area. To locate a clandestine grave, several techniques can be applied to find the physical and chemical changes that are caused by the decomposition of the buried corpse (Hopkins et al. 2000; Hunter and Cox 2005; Carter et al. 2008; Tibbett and Carter 2008; Van Belle et al. 2009; Pringle and Jervis 2010; Pringle et al. 2010). During a field search, experts in the areas of forensic archaeology and forensic anthropology are very closely involved. Forensic archaeology mainly involves; location and recovery of (clandestine) graves where forensic anthropology mainly focuses on the investigation of the human skeleton. A discipline that provides the overlap in these two areas is forensic taphonomy. Forensic taphonomy is an applied expertise that focuses on the reconstruction of events before, during and after the death (Broeders and Muller 2008; Tibbett and Carter 2008).

For the reconstruction, material of the natural environment is collected in the grave or in the vicinity of the cadaver. During the scene of crime investigation of a clandestine grave, several traces can be found in the form of botanical (plant residues, pollen etc. (Gunn 2006), geological (minerals, rocks, including remains of earth in different shape and texture (Hunter and Cox 2005; Campbell et al. 2008; Tibbett and Carter 2008), and entomological traces (insects in various stages of development (Gunn 2006). Using a field based search combined with laboratory techniques, an indication can be given of how long someone has been deceased, also an estimation can be made as to whether or not the decomposition has occurred in the localised grave. During the field investigation numerous (geo-)scientific techniques are applied in the form of destructive and non destructive techniques. The techniques mainly used are; soil probing, cadaver dogs and excavations. Also non-destructive field mapping techniques can be used in the form of thermal cameras (infrared red), metal detectors, remote sensing (aerial photography) and ground penetrating radar (GPR) (Pringle et al. 2012). Each method has its own advantages and disadvantages that still may be specific to the target location (Hunter and Cox 2005; Broeders and Muller 2008; Tibbett and Carter 2008; Pringle and Jervis 2010; Pringle et al. 2010; Swann et al. 2010).

The surface of the earth can be divided into different layers. These layers are very important for the maintenance of life on earth. The topsoil is the uppermost layer and consists of a very complex ecosystem. The complexity of this ecosystem is formed over time by climatic, mineralogical, biological, chemical and physical conditions (Swann et al. 2010). The topsoil is mainly composed of organic and inorganic components, such as; soil, plants, bacteria and fungi, which all play a major role in the formation of the nitrogen cycle (Van der Schoot and Leegwater 2005;

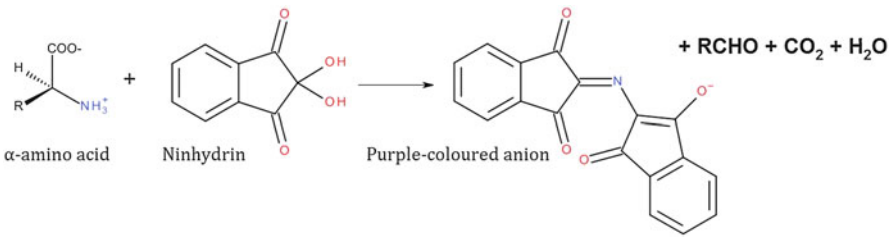

Fig. 15.1 Chemical reaction of ninhydrin reagent with an α-amino acid, two ninhydrin molecules bind with the nitrogen group, forming a purple-coloured anion (Ruhemann's purple), an aldehyde, CO_2 and water (According Wade (2009))

Johll 2007; Campbell et al. 2008). The main sources of nitrogen on earth are; the atmosphere (N_2), the biomasses of living organisms (nitrogenous organic compounds in the form of; amino acids, proteins, deoxyribonucleic acid (DNA) and chlorophyll) and surface areas such as; soil, sediments, lakes rivers and oceans (Campbell et al. 2008). When animals or humans die, the physical and chemical composition of their bodies will change completely and ammonifying bacteria, which live in the humus-rich topsoil, covert the releasing organic nitrogen compounds in forms of proteins, (poly-)peptides and amino acids into nitrogenous minerals. Besides the influence of ammonifying bacteria, also environmental influences, climate changes and the attraction of other organisms affect the decomposition process (Dent et al. 2004; Forbes et al. 2005; Gunn 2006; Tibbett and Carter 2008).

Two research studies (Carter et al. 2008; Van Belle et al. 2009), have shown that ninhydrin could be an useful chemical reagent for the determination of these nitrogenous minerals, in the form of; proteins, (poly-)peptides, amino acids and ammonium (NH_4^+) in soil. These nitrogenous minerals are therefore better described as ninhydrin reactive nitrogen (NRN). Ninhydrin is a well recognised reagent in forensic science for the detection of latent fingerprints on (porous-) surfaces such as paper and cardboard (Odén and von Hofsten (1954). The reaction mechanism of ninhydrin manly involves a reaction with α-amino acids. α-Amino acids contain a primary amino group that can react with ninhydrin to form a binding site of two ninhydrin molecules with the nitrogen group. This binding of two ninhydrin molecules with a nitrogen group results in a purple-coloured anion known as Ruhemann's purple including an aldehyde, CO_2 and water (Fig. 15.1) (Wade 2009). In cases where it is suspected that a clandestine grave is found, a 'rapid on the scene' method can be useful to indicate whether or not a further (chemical-/biological) investigation is needed. This study shows a possible presumptive test that can be used before or after forensic geo-scientific techniques to contribute to the detection of possible decomposition in clandestine graves, while presenting a new application of the ninhydrin reagent.

15.2 Experimental

15.2.1 Reagents and Standards

The amino acid analytes phenylalanine and D-leucince were obtained from Fisher Scientific (Landsmeer, the Netherlands) and Acros Organics (Geel, Belgium) respectively. Pre-prepared ninhydrin reagent was purchased from BVDA (Haarlem, the Netherlands), solid ninhydrin reagent and hydrindantin hydrate were both acquired from Sigma Aldrich (Steinheim, Germany). The chemicals dimethyl sulfoxide and sodium acetate, were both ordered from Boom laboratory supplies (Meppel, the Netherlands). Masonry sand was purchased from a local do it yourself-shop Karwei (Breda, the Netherlands) and all aqueous standards and reagents were prepared by using deionised water obtained from the laboratory.

15.2.2 Decomposition Samples

Research samples were a kind gift from the *University of Central Lancashire*, Preston UK. Soil samples were taken in a $n = 1$ replicate with a total of twelve soil samples ($n = 12$). A pig *(Sus domesticus)* was laid down on the soil surface and samples were collected in the direct vicinity (~10 cm) or under the cadaver, after a decay period of 2 months and placed into falcon tubes of 50 mL. Samples were all labelled following the position collected; hind leg, head and abdomen, including the 12, 6 and 9 o'clock positions (Labelled code was following digital clock time; e.g. C16 18.00, pig code 6 o'clock position) (Fig. 15.2). The soil samples were then posted on dry ice, and upon arrival in the Netherlands immediately stored at −70 °C. Control soil samples were all collected in the area of Breda (NL) and spiked control samples were prepared by mixing 3.5 and 43.0 mg solid phenylalanine into 10.0 mg of soil.

15.2.3 Filter Paper Method

In the research articles of Carter et al. (2008) and Van Belle et al. (2009), the usage of ninhydrin as detection reagent for decomposition was conducted as a quantitative measurement of NRN using spectrophotometry. Inspired by this approach, the method described in this paper uses a simplified application that can be used as a qualitative measurement for the detection of NRN. The filter paper method was carried out by spraying deionised water onto a soil samples and adsorbing the moisture onto a cellulose filter paper from Schleicher & Schuell, Ø70 mm (Shifferstadt, Germany). The filter paper was subsequently sprayed with pre-prepared ninhydrin

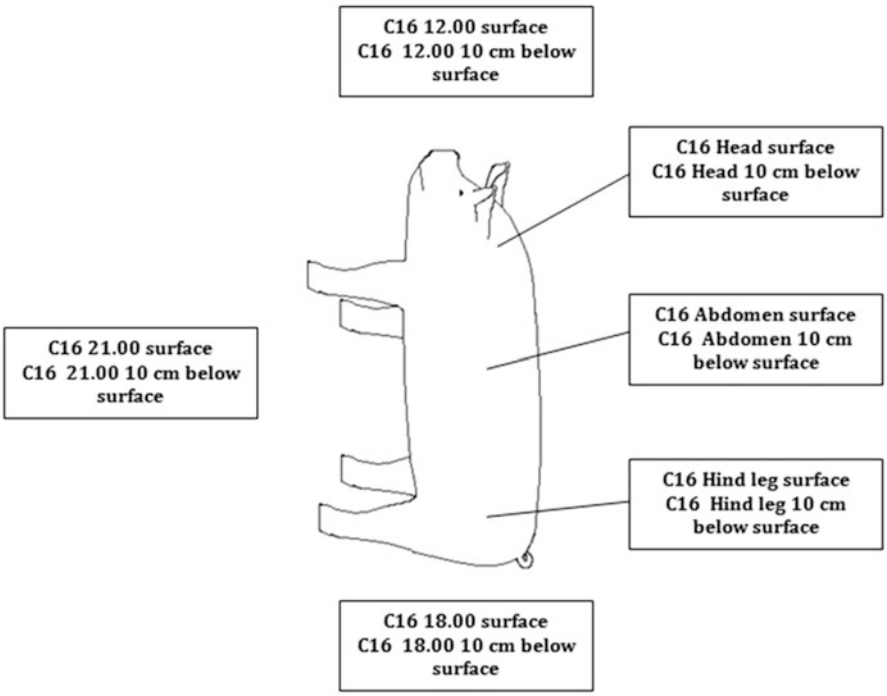

Fig. 15.2 Sample coding for single taken soil samples $n = 1$, with a total of $n = 12$. The samples C16 12.00, 18.00 and 21.00 o'clock positions are taken in the direct vicinity, ~10 cm away, of the decomposing *Sus domesticus*. C16 head, abdomen and hind leg samples are taken directly under the decomposing pig after a decay period of 2 months

Fig. 15.3 Schematic representation of the new ninhydrin application, where the soil is moisturised using deionised water. A filter paper is than pressed onto the moisturised soil and subsequently sprayed with ninhydrin reagent. The treated filter paper was allowed to dry and then placed for approximately 10 min at 80 °C in an oven, so the result could be interpreted afterwards

reagent (ninhydrin dissolved in ethanol, acetic acid, petroleum ether and MTBE), and incubated for approximately 10 min at 80 °C (Fig. 15.3). Prior to applying the ninhydrin reagent to real decomposition samples, positive and negative controls were carried out for the pre-prepared ninhydrin reagent, using fingerprint-touched and untouched filter papers.

15.2.4 Filter Paper Development

Further development of the ninhydrin reagent and application of the qualitative technique was done by testing different filter paper approaches such as; ninhydrin saturation, ninhydrin spraying and ninhydrin pipetting. Modified ninhydrin reagent was freshly prepared before each experiment following the method described by Van Belle et al. (2009). To summarise this method, 800 mg of ninhydrin and 120 mg of hydrindantin hydrate, were weighed and dissolved in 30 mL of dimethyl sulfoxide and 10 mL of 4 M-sodium acetate. The prepared ninhydrin reagent was then covered by aluminium foil and allowed to stand at 4 °C until analysis.

A simulation study was carried out using different concentrations; 20, 40 and 80 µg/mL of freshly prepared D-leucine stock standard (500 µg/mL), spotted onto a filter paper and simulation soil using masonry sand.

15.3 Results and Discussion

15.3.1 Result Interpretation

Colour developments of presumptive test results are very subjective and therefore the interpretation can be biased or inaccurate. To simplify and classify the colour change interpretation, different criteria are established and given in Table 15.1.

In order to determine the test results, the simulation study with D-leucine standard have resulted in an accurate representation where colour change is correlated to amino acid concentration (Fig. 15.4). Supported by the results described in the papers of Carter et al. (2008) and Van Belle et al. (2009) it is shown that colour intensity will increase in proportion to higher presence of amino acids (Fig. 15.4). However, it is noteworthy that positive results observed after testing 2 month old decomposition samples (Fig. 15.5), show a decreased intensity in deeper soil samples. This difference in intensity is according the concentration amino acids transferred into the filter paper. Looking at the samples; C16 Abdomen 10 cm below

Table 15.1 Useful criteria for the result determination

Score	Description	Colour
−	No colour change	Nothing visible (0)[a]
+−	Average colour change	Rose-light purple (5RP 5/10) [a]
+	Good colour change	Purple (5P 5/10)[a]
++	Excellent colour change	Purple- deep purple (5 PB 5/10) [a]

[a]Interpretation of the colours done according Munsell colour chart, using the hues; Red (R), Yellow (Y), Green (G), Blue (B) and Purple (P), including the lightness and brightness of the colour (the value and chroma)

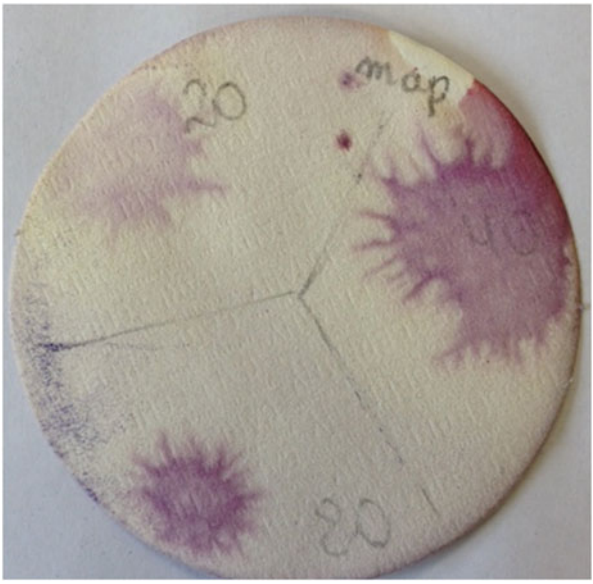

Fig. 15.4 Filter paper showing 20, 40 and 80 μg/mL of D-leucine spots, the paper was pre-treated with ninhydrin reagent before spotting the amino acid

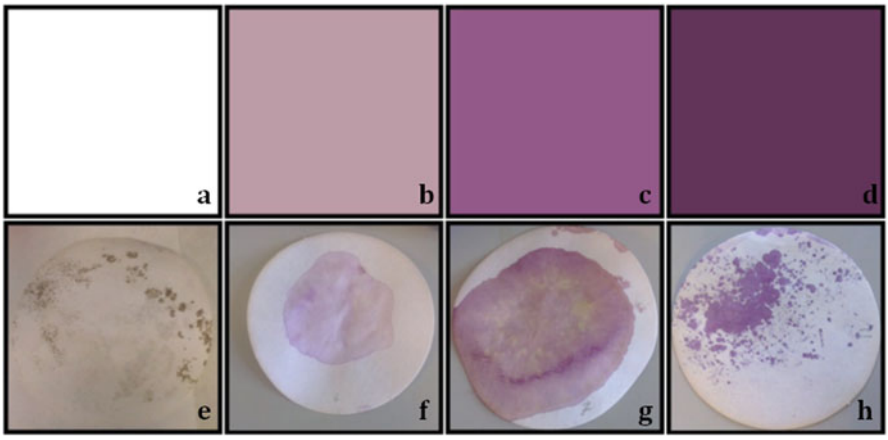

Fig. 15.5 A theoretical colour chart (**a, b, c, d**) compared to positive results obtained from: (**e**) Blank soil sample; (**f**) C16 Abdomen 10 cm below surface; (**g**) C16 Abdomen surface; (**h**) Control sample spiked with 43.0 mg phenylalanine in 10.0 g soil

surface and C16 Abdomen surface, a light purple colour and strong purple colour development are observed (Fig. 15.5f and g). Comparing these results with the filter paper showing different concentrations of known D-leucine standard (Fig. 15.4), it is demonstrated that the amino acid presences for the C16 Abdomen 10 cm below surface (Fig. 15.5f) is lower than C16 Abdomen surface. Nonetheless, it should be mentioned that the applied test is indicative for the presence of amino acids, and

therefore no actual conclusion can be made about the concentration amino acids present, mainly since no quantitative measurement is carried out to confirm this observation.

15.3.2 Decomposition Samples

Several control and decomposition samples were investigated while using the ninhydrin spray test application. During the test, it became clear that the negative controls had not reacted with the ninhydrin reagent. The prepared spiked control samples indicated on the other hand, a very strong purple colour change. This colour change can be interpreted following Table 15.1 as 'excellent colour change'. Also the tested decomposition samples have shown positive colour indications which ranged from 'average colour change' to 'good colour change'. Mainly the soil samples taken at the surface areas; head, abdomen and hind leg showed a positive indication for the presence of amino acids. The samples taken on these areas 10 cm below the surface, resulted in an 'average colour change' or even in a negative test result, Table 15.2, which indicates that the presence of amino acids derived from decay is lower for areas less close to the actual decomposition source.

However it is found that ninhydrin indicates a positive test result in the presence of amino acids, it should be noted that amino acids do occur naturally in soil

Table 15.2 Test results control- and decomposition samples

Sample code	Result
Blank filter paper (negative control)	–
Blank soil sample (negative control)	–
Filter paper with fingerprint (positive control)	+
3.5 mg phenylalanine in 10 mg soil (spiked control)	++
43.0 mg phenylalanine in 10 mg soil (spiked control)	++
C16 12.00 surface	+–
C16 12.00 10 cm below surface	+–
C16 Head	+
C16 Head 10 cm below surface	–
C16 Abdomen surface	+
C16 Abdomen 10 cm below surface	+–
C16 Hind leg surface	+
C16 Hind leg 10 cm below surface	+
C16 18.00 surface	+–
C16 18.00 10 cm below surface	+–
C16 21.00 surface	+
C16 21.00 10 cm below surface	+–

(Sowden and Ivarson 1966; Campbell et al. 2008; Jämtgård 2009). Also since there is no quantitative test carried out as a control measurement for the presence of naturally occurring amino acids, it is impossible to conclude whether these compounds have contributed to the ninhydrin reaction. Also important to notice is that the control soil samples taken in Breda (the Netherlands) do not show any colour change, which might indicate that amino acids can be present in low concentrations beneath the visual limit of detection of the ninhydrin reagent.

It must be taken into account although the applied method yielded a positive result for some decomposition samples it is still presumptive in nature. Application of the current method will depend on the circumstances of the case, and evidential value will be increased when applying this current described method previously or afterwards in combination with other existing techniques to determine decay.

15.3.3 Filter Paper Development

During the filter paper development using D-leucine standard 80 μg/mL, three different approaches were carried out to determine the best visible test result on a cellular filter paper. In the first method D-leucine standard was directly pipetted onto a clean filter paper and treated with ninhydrin reagent while dropping it onto. This application shows that D-leucine present on the filter paper is likely to migrate. This migration arises in a poor observable test result, since the colour change is less identifiable and appears as a big blurred spot. Also the spraying method, previously described in this paper was evaluated, and resulted in a spatter pattern. This spatter pattern occurs since the ninhydrin reagent seems not to be equally distributed and could be difficult to interpret, since there is no homogenous colour development present. However it is thought that this can be overcome by using a better spray bottle or airbrush to distribute the ninhydrin reagent more dense. A third application method gave the most optimal test results, since the spots of D-leucine were added in the centre of the filter paper onto a pre-treated ninhydrin reagent filter paper. As shown in the results (Figs. 15.4 and 15.6), the spots with D-leucine standard are less likely to migrate through the paper and the colour observation of the different shades

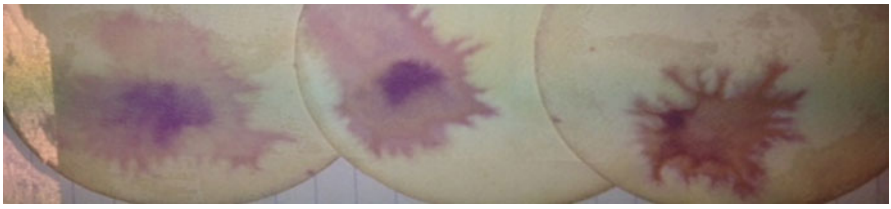

Fig. 15.6 Three filter papers are saturated in modified-ninhydrin reagent, dried at room temperature, and later used to pipette directly D-leucine amino acid standard onto the pre-prepared filter papers. Colour development will occur after placing the paper in a oven at 80 °C for approximately 10 min

of purple are easier and more clearly visible than in the other used methods. Since the filter paper saturation is only carried out with D-leucine standard, it is important to mention, that further analysis is needed for the detection of NRN present in complex matrices.

15.4 Conclusions

Ninhydrin reactive nitrogen (NRN) compounds are released into soil during the decomposition of a corpse. To demonstrate NRN compounds directly in soil, a new application form of the well known ninhydrin reagent was successfully developed. The emerging method used filter paper adsorption from moisturised soil samples prior to the colour reaction and was applied to soil and gravesoil. Results were determined by interpretation of the purple colour change and have shown that ninhydrin is a useful reagent for the detection of decomposition.

Also further development of the pre-treated filter paper, saturated in ninhydrin reagent could be useful for the detection of NRN, since results have shown that NRN compounds are less likely to migrate through the paper.

Since the nitrogen cycle will bring the value of NRN compounds back to a normal value (Van der Schoot and Leegwater 2005; Campbell et al. 2008, it is worth to mention that the concentration of NRN could fall beneath the limit of detection, and therefore this test application could be out of use in some cases. To overcome these circumstances the usage of other amino-acid reagents might be considered.

The ninhydrin test presented in this paper, used at the realistic decomposition samples, is still a draft for the development of a 'rapid on the scene test' for the detection of suspected clandestine graves. After method validation, the designed concept method could be a complementary test to the current used testing approaches, such as; cadaver dogs, puncture probe and Ground Penetrating Radar. The results have shown that no sophisticated extraction methods are required and that the colour development will appear within 10 min after using an oven. Also it is important to mention that this technique is relatively cheap in comparison to other methods and advanced detectors.

Published by previously research studies (Hopkins et al. 2000; Carter et al. 2008; Van Belle et al. 2009; Pringle and Jervis 2010; Pringle et al. 2010, 2012) it is shown that the decomposition of domestic pigs is a good model for human decay. As described by Van Belle et al. (2009), the adult *Sus domestica* is equal to a human torso by biochemistry, physiology and fat to muscle ratio. The used decomposition samples provided by the University of Central Lancashire have shown the release of NRN compounds, since these are detected by using the new ninhydrin application form. After this positive test result it is still important to mention that the technique described in this study, is just an indicative test. To confirm the obtained colour change, it is vital to use more advanced (analytical laboratory) confirmation techniques.

To summarise; the analysis of ninhydrin reactive nitrogen in decomposition samples is possible after 2 months of decomposition. Also the first results have shown that further development of the ninhydrin saturated filter paper may lead to a point of care testing paper, which can provide an indicative test result.

15.5 Recommendation-Future Perspective

The development of this new application of the ninhydrin reagent, described as in this study, is still going on at Avans University of applied sciences. At the moment different research approaches are carried out in order to validate the method within the forensic standard recommendations.

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