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DOI
10.1016/j.forsciint.2018.03.019

Publication date
2018

Document Version
Final published version

Published in
Forensic Science International

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Citation for published version (APA):
Case Report

Wastewater-based epidemiology generated forensic information: Amphetamine synthesis waste and its impact on a small sewage treatment plant

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A R T I C L E   I N F O

Article history:
Received 2 September 2017
Received in revised form 18 January 2018
Accepted 9 March 2018
Available online 19 March 2018

Keywords:
Amphetamine synthesis waste
Non-target screening
Domestic wastewater treatment plant
Pre-precursor APAA
1-Phenylpropan-2-one
Synthesis by-products

A B S T R A C T

Chemical analysis of domestic wastewater can reveal the presence of illicit drugs either consumed by a population or directly discharged into the sewer system. In the search for causes of a recent malfunctioning of a small domestic wastewater treatment plant aberrantly high loads of amphetamine were observed in the influent of the plant. Direct discharges of chemical waste from illegal production sites were suspected to be the cause. Illegal manufacturing of amphetamines creates substantial amounts of chemical waste. Here we show that fly-tipping of chemical waste originating from an amphetamine synthesis in the catchment of a small sewage treatment plant resulted in failure of the treatment process. Target analysis of drugs of abuse and non-target screening using high resolution mass spectrometry provided evidence for the presence of amphetamine produced from the precursor 1-phenylpropan-2-one by the Leuckart process through specific synthesis markers. Furthermore the identity and presence of the pre-precursor 3-oxo-2-phenylbutanamide was confirmed and a route specific marker was proposed. This is the first study that demonstrates that non-target screening of wastewater can identify intermediates, impurities and by-products of the synthesis routes used in illegal manufacturing of amphetamine. The profiles of chemicals thus obtained can be used in tracking productions sites within the corresponding sewer catchment.

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1. Introduction

The principle of using chemical analysis of wastewater to determine the use of illicit drugs in a population, also known as wastewater-based epidemiology (WBE), was first demonstrated in 2005 [1]. Since then WBE has evolved to an acknowledged technique and been widely applied. The first European-wide survey to compare consumption data of city populations on the basis of wastewater loads was performed in 2011 [2] simultaneously in 19 cities, followed by an even larger number of cities in the following years [3]. Castiglioni et al. [4] addressed the uncertainties associated with all the steps used to estimate community drug consumption from the chemical analysis of sewage biomarkers of illicit drugs. However, some incidents have been reported where aberrantly high loads of drugs of abuse were observed in sewers that could not be ascribed to human consumption only [5]. In 2014 for the first time it was reported that aberrant levels of MDMA observed in the influent of a city in The Netherlands did not originate from consumption. By determining the ratios of MDMA enantiomers in the influent a racemic composition was found to be present in the wastewater [6] reflecting direct disposal of non consumed MDMA into the sewer system. In that study it was hypothesized that a dump of MDMA tablets or pure material had taken place under the pressure of a police raid. During the European studies performed since 2011 [7] every year in the influent of the Eindhoven WWTP in The Netherlands aberrantly high amphetamine levels were encountered relative to what can be considered “normal”, consumption-related loads of amphetamine [3]. Such loads cannot possibly be explained by consumption only. The most likely origin of these high loads are direct dumps of synthesis waste originating from the production of amphetamine. No effects on the treatment capacity of the corresponding WWTP were noticed by the operators. The WWTP of Eindhoven is one of the largest in the Netherlands with a capacity of 750,000 inhabitant equivalents (i.e.) and a hydraulic...
capacity during dry weather of 10,000 m³/h and 30,000 m³/h during rainy weather. Its buffering capacity is much larger than that of a smaller sewage treatment plant (<20,000 i.e.). Both the European Monitoring Centre for Drug and Drug Addiction [8] and the United Nations Office for Drugs and Crime [9] have pointed at the link between illegal production and disposal of synthesis waste in the environment implicating that not only the human health risks but also the potential impact on the environment is a reason for concern. Specifically hazardous and toxic wastes from synthetic drug production (by-products and unused chemicals) are frequently disposed of in urban sewerage systems [8,9].

1.1. Amphetamine synthesis

Amphetamine is one of the most popular synthetic drugs in a crowded stimulant market in Europe [8]. The Netherlands and Belgium are accounting for a great part of its production in Europe [8,10]. The most common synthesis used in the production of amphetamine is the Leuckart synthesis because of its relative simplicity. In three straightforward steps the precursor BMK (BenzyloMethylKetone, 1-Phenyl-2-propanone, [CAS 103-79-7]) is transformed into amphetamine. BMK is a controlled chemical in the EU drug precursor chemical legislation since 2004 [11]. Producers are quite inventive to circumvent the legal restrictions of importing and producing precursors. In 2008 the first laboratory was discovered in the Netherlands were BMK-bisulphite salt [960059-22-7] was encountered [12]. This is a so called masked pre-cursor (non-controlled) which is very easily transformed into BMK. In 2010 for the first time the pre-precursor APAAN (Alpha-PhenylAcetoacetateNitrile or 3-oxo-2-phenylbutanenitrile [4468-48-8]) was encountered by a joint effort of the Belgium customs and the Dutch police [12]. At the end of 2013 APAAN was included in the existing EU precursor legislation [13]. Next, BMK-methyl glycidate [80532-66-7] or the BMK Glycidic Acid [5449-12-7] were the newly designed masked precursors, which can also be easily transformed into BMK. In 2013 a pre-pre-precursor was discovered in the Netherlands 3-oxo-N-phenylbutanamide [102-01-2] which can be readily converted into APAAN [8]. Then followed in 2015 by the discovery of containers with the starting material APAAN (Alpha-PhenylAcetoacetamide) or 3-oxo-2-phenylbutanamide [4433-77-6][8], which were seized in Poland and Germany in 2015 for the first time. APAAN can be readily transformed into BMK. Still more and pre-precursors are emerging as a recent internet search showed that, 3-oxo-4-phenyl-butyric acid ethyl ester [718-08-1], 3-oxo-2-phenylbutanoic acid [4433-88-9], methyl-3-oxo-2-phenylbutyrate [16648-44-5] are advertised as BMK-intermediates (see Fig. 1).

A side effect of the conversion of chemicals to a (pre-)precursor is the extra production step. They need to be hydrolysed with a strong concentrated acid (e.g. sulphuric acid) to obtain BMK. The Leuckart synthesis BMK to Amphetamine alone generates already an estimated 20–30 kg of chemical synthesis waste per 1 kg of amphetamine [8]. In this process an intermediate (N-formylamphetamine) is formed by heating BMK with formamide in the presence of formic acid. The intermediate needs to be hydrolysed again with a strong acid. After neutralization the pure amphetamine oil is treated with sulphuric acid to obtain the crystalline form. All these steps generate acidic synthesis waste, of which producers have to get rid. The most common way of fly-tipping is disposing the waste in the environment in closed containers [8],

![Fig. 1. (Pre-)Precursors available for synthesizing amphetamine through the Leuckart process.](image-url)
but increasingly, alternative ways of fly-tipping have been reported. Since 2010 the number of dumps registered by the police in The Netherlands has been increasing rapidly from 32 to 177 in 2016 [14]. However, due to the frequency of occurrence and the efforts needed to determine if a dump is really originating from synthesis waste it is believed that this is a just fraction of the total number of dumps [14]. Other ways of disposal include direct dumping in sewers, and recently it was shown that mixing waste with manure is also practised and, when used to fertilize farmland, results in ppb traces of amphetamine ending up in corn [15].

The purpose of the present study was to (i) investigate the wastewater collected from a malfunctioning WWTP by employing high-resolution accurate mass spectrometry (HRMS) through a target mass spectrometric screening on illicit drugs and a non-target screening, and (ii) to explore if the origin of the waste could be backtracked on the basis of pre-cursors and synthesis by-products identified by the non-target screening.

2. Materials and methods

2.1. Sample collection and preparation

Three different samples were collected. The wastewater sample of 2016 was a grab sample taken directly from the aeration tank of the failing WWTP. From the 2017 incident a grab sample of wastewater was taken out of a pumping station located 2 km upstream from the WWTP. A solid sample was taken out of a 25 kilo bag found at the discovered amphetamine manufacturing location containing a crystalline yellow substance. The wastewater samples were spiked with deuterium-labeled internal standards, adjusted to pH 7.0 with NaOH, and filtered. 100 mL of the filtered extract were concentrated by solid phase extraction on Oasis-HLB cartridges. The solid sample was dissolved in methanol and diluted to 100 µg/L in ultra-pure water.

For the screening samples were analysed with SPE-HPLC-DAD-LTQ-Orbitrap-MS. More extensive details can be found elsewhere [5,16]. The non-target screening was performed by transferring the adjusted and filtered sample directly to the vial and injecting an aliquot onto a HPLC-Orbitrap-Fusion. A suspect list was created to be used in post processing of the non-target data for suspect screening. More information on the methodologies can be found in the supplementary information.

2.2. Case description

In 2016 at a small WWTP (16,000 i.e., maximum capacity of 460 m³/h) in the south of The Netherlands the ammonia levels rose, causing the aeration to respond automatically, to no avail: levels of ammonia did not decrease. A nitrification test on the active sludge showed that all nitrifying bacteria were inactive (ated). This resulted in a failure of the WWTP and a release of untreated wastewater into a small receiving stream. The acidity values recorded in the influent were decreasing to a pH of 2 and enforcement officers of the water board tried to locate the origin of the possible pollution, simply by measuring the pH in the sewage system. Heavy rainfall prevented the final localization of the source. After three days the active sludge culture in the original WWTP was newly inoculated and the treatment returned to normal. In January 2017 the pH of the influent of the same WWTP decreased again. Immediately a high pressure pipe coming down from an area suspected to contain the origin of the waste was shut down. By sampling and measuring the pH in the wastewater of the corresponding local pumping station it became evident that the origin of the low pH was in this part of the catchment area. A suspect and non-target screening was commissioned subsequently on samples from the WWTP. The next day the police discovered an illegal manufacturing location for the production of amphetamine. The site was topped with both empty and filled containers of laboratory synthesis waste (an estimated 10,500 L). Also a large number of empty and filled bags were found with solid substances that presumably (had) contained (pre)-precursors for the synthesis of amphetamine. The location was found to be connected directly to the sewer.

3. Results and discussion

3.1. Malfunction of the WWTP

The wastewater sample collected in 2016 was highly different from usual, with a cloudy and almost milk-like appearance. The pH of the sample was 2.0. The final extract had a dark red brown color. The January 2017 wastewater sample had a pH of 1.0 and a similar cloudy and almost milk-like appearance. The high acidity levels are toxic for the nitrifying bacteria in the active sludge since these are very sensitive [17] especially to disturbances away from the optimum pH of 7.8 [18]. At these low pH all nitrifying bacteria are inactivated, and for the WWTP to return back to normal, the sludge needed to be inoculated with a fresh culture. In the incident in April 2016 an estimated 175 m³ of fresh active sludge was inoculated corresponding to about 40% of the total amount of sludge for this WWTP.

3.2. Amphetamine/benzoylecgonine ratios

The influent samples collected were subjected to a target mass spectrometric screening focussing on amphetamine and benzoylecgonine. In The Netherlands, in urban wastewater not impacted by direct discharges, as in the cities of Amsterdam and Utrecht, the ratios of concentrations of amphetamine and benzoylecgonine (a biomarker for cocaine consumption) usually are less than one (see Fig. 2).

A statistically significant difference (95% confidence interval) for the distribution (five year) of the ratios (Kruskal–Wallis

![Fig. 2. Ratios of amphetamine (µg/L) and benzoylecgonine (µg/L) in three WWTPs in The Netherlands: Eindhoven, Amsterdam and Utrecht (2011–2015, n = 7 except where indicated) and in the case study WWTP. Values above 1 indicate abnormal ratios.](image-url)
where average ratios (0.56 vs 0.48) were not significantly different (t-test p > 0.29). The results of the present target screening revealed that in the case of the wastewater collected in April 2016 at the malfunctioning plant this ratio amounted to 8, whereas in

Table 1
List of suspects used in the non-target screening for amphetamine synthesis-specific impurities, intermediates, by products and final product

<table>
<thead>
<tr>
<th>Retention time</th>
<th>Accurate mass</th>
<th>Bruto formula</th>
<th>Identification Level</th>
<th>Compound</th>
<th>Function</th>
<th>Occurence in sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>Standard</td>
<td>APAA</td>
<td>[M+H]+</td>
<td></td>
<td></td>
<td>2016</td>
</tr>
<tr>
<td>5.02</td>
<td>150.09134</td>
<td>C₉H₁₁NO</td>
<td>4</td>
<td>1-Phenyl-2-propanone oxime</td>
<td>6</td>
<td>√</td>
</tr>
<tr>
<td>6.04</td>
<td>158.09642</td>
<td>C₁₁H₁₃N</td>
<td>3</td>
<td>2-Phenylacetamide</td>
<td>6</td>
<td>√</td>
</tr>
<tr>
<td>6.47</td>
<td>164.10099</td>
<td>C₁₁H₁₂N</td>
<td>3</td>
<td>N-Formylamphetamine</td>
<td>4</td>
<td>√</td>
</tr>
<tr>
<td>6.71</td>
<td>136.11208</td>
<td>C₈H₁₀NO</td>
<td>1</td>
<td>Amphetamine</td>
<td>3</td>
<td>√</td>
</tr>
<tr>
<td>7.89</td>
<td>136.07569</td>
<td>C₇H₁₁NO</td>
<td>3</td>
<td>2-Phenylacetamide</td>
<td>6</td>
<td>√</td>
</tr>
<tr>
<td>8.70</td>
<td>178.08626</td>
<td>C₁₀H₁₃NO₂</td>
<td>2</td>
<td>APAA (Alpha-Phenylicetoacetamide) Keta</td>
<td>1</td>
<td>√</td>
</tr>
<tr>
<td>9.74</td>
<td>212.14337</td>
<td>C₁₀H₁₂N</td>
<td>3</td>
<td>α-Benzylphenethylamine(dibenzylmethylamine)</td>
<td>6</td>
<td>√</td>
</tr>
<tr>
<td>10.01</td>
<td>178.08626</td>
<td>C₁₀H₁₃NO₂</td>
<td>1</td>
<td>3-oxo-N-phenylbutanamide</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>11.20</td>
<td>171.09167</td>
<td>C₁₀H₁₂N</td>
<td>3</td>
<td>4-Benzylpyrimidine</td>
<td>5</td>
<td>√</td>
</tr>
<tr>
<td>12.73</td>
<td>171.09167</td>
<td>C₁₁H₁₂N</td>
<td>3</td>
<td>5-Fenyl-4-methylpyrimidine</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>12.98</td>
<td>254.19032</td>
<td>C₁₂H₁₄N</td>
<td>3</td>
<td>D-[(β-phenylisopropyl)amine</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>13.13</td>
<td>135.08044</td>
<td>C₁₁H₁₀O</td>
<td>1</td>
<td>BMK (BenzylMethylketone)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>13.66</td>
<td>178.08626</td>
<td>C₁₀H₁₃NO₂</td>
<td>2</td>
<td>APAA (Alpha-Phenylicetoacetamide) Enol</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>13.96</td>
<td>160.07569</td>
<td>C₁₀H₁₂NO</td>
<td>1</td>
<td>APAA (Alpha-Phenylicetoacetamide)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>14.29</td>
<td>277.13382</td>
<td>C₁₀H₁₀N₂</td>
<td>4</td>
<td>Unknown</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>16.91</td>
<td>276.13829</td>
<td>C₁₀H₁₂NO</td>
<td>3</td>
<td>4,6-Dimethyl-3,5-diphenylpyridin-2-one</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>18.62</td>
<td>18.61</td>
<td>C₁₀H₁₂NO₂</td>
<td>3</td>
<td>2,3-Diacetyl-2,3-diphenylsuccinonitrile</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

a Functions: 1. Pre-precursor; 2. precursor; 3. main product; 4. Leuckart intermediate; 5. Leuckart route specific impurity; 6. marker route APAA — BMK — AMP; 7. marker route APAA — BMK — AMP

b IUPAC name: 3-oxo-2-phenylbutanamide.

Fig. 3. Base peak chromatogram (115–1300 m/z) of the wastewater sample collected in April 2016 (top) and corresponding total scan Photodiode array (190–800 nm) chromatogram (bottom).
January 2017 it had amounted to 50. These were the first indications that non-consumed amphetamine had been discharged directly into the corresponding sewer.

### 3.3. Non-targeted mass spectrometric screening

Besides targeted screening with HRMS it offers the possibility to investigate the presence of many other compounds [19], by using the basepeak chromatogram and using a suspect list with accurate masses (Table 1) in a non-target screening mode. The first signs of a large scale pollution with anthropogenic substances were observed in the base peak chromatogram of the Orbitrap. Very intense signals were encountered, and these were much larger than those of homologue polyethylene glycol (PEG) series (see Fig. 3) normally found in the base peak chromatogram of a wastewater sample. The corresponding diode array (DAD) chromatogram was also suggesting a high abundance of aromatic compounds (see Fig. 3).

The most intense signal (Rt 15.67 min) in the accurate mass chromatogram of the wastewater samples belonged to the protonated molecular ion from \( \text{C}_{10}\text{H}_{11}\text{N}_{2}\text{O} \) \([\text{M}+\text{H}]^+\) m/z = 277.13353. The number of carbon and hydrogen atoms suggests a large aromatic structure. The corresponding chromatographic peak in the DAD signal showed no absorption in the visible wavelength (see insert Fig. 3) ruling out the most likely candidate for \( \text{C}_{18}\text{H}_{16}\text{N}_{2}\text{O} \): SUDAN II [3118-97-6], a colorant. Further investigations need to be undertaken to determine the identity of this peak.

The non-target screening, based on the suspect list showed the presence of BMK (Table 1) in the sample which was confirmed by comparison with a standard solution. There is a route-specific impurity associated with the Leuckart synthesis from BMK to amphetamine: 5-phenyl-4-methylpyrimide (5P4M) first proposed by van der Ark et al. [20] and later confirmed by Kirkride et al. [21]. After retrospective analysis of the raw data file a signal was discovered corresponding to the accurate mass of 5P4M. An analytical standard of 5P4M was not available, but the accurate fragmentation spectrum was compared with the predicted one (Fig. 1 S.I.) and matched perfectly.

While this synthesis marker strongly suggests the Leuckart route being used, it does not enable a distinction of which precursor was used.

BMK is controlled in Europe [11] and therefore most likely produced from a precursor. The most likely precursor would be APAAN [8]. In the raw data file a signal was encountered for the m/z value of APAAN but its identity could not be confirmed because the retention time (difference ~0.82 min) (see Table 1) and fragmentation spectrum were not matching with a standard solution. After closely examining the signals recorded (see Fig. 4).

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**Fig. 4.** Extracted ion chromatograms of the wastewater sample collected in 2016 (top left: showing the protonated molecular ion \( \text{C}_{10}\text{H}_{11}\text{N}_{2}\text{O} \) APAAN; middle, showing the protonated molecular ion \( \text{C}_{10}\text{H}_{11}\text{N}_{2}\text{O} \) losing a water molecule; bottom, showing the protonated molecular ion \( \text{C}_{10}\text{H}_{11}\text{N}_{2}\text{O} \) losing an ammonia molecule; right hand panel, the accurate mass spectrum at 13.65 min).
It was observed that the protonated molecular ion with the most likely bruto formula $\text{C}_6\text{H}_{12}\text{N}_2\text{O}_2$ ([M+H]$^+$) $m/z = 178.08625$ is losing water by in source dissociation which is indicative of a compound containing a hydroxyl group. As a result, a signal appears with the same $m$/z value as the probable molecular ion for APAAN [$\text{C}_6\text{H}_{12}\text{N}_2\text{O}_3$]$^+$ v.s. [$\text{C}_6\text{H}_{12}\text{N}_2\text{O}_2 + \text{H}_2\text{O}]^+$. Interestingly, signals were observed for two impurities which are claimed [22] to be specific for the hydrolysis of APAAN to BMK [22], namely 4,6-dimethyl-3,5-diphenylpyridine-2-one [m/z 276.13829] and 2-phenylacetamide [m/z 136.07569]. The chemical formula $\text{C}_6\text{H}_{12}\text{N}_2\text{O}_2$ observed corresponds with two possible pre-precurors: 3-oxo-2-phenylbutanamide or 3-oxo-N-phenylbutanamide. In both wastewater samples we encountered two large signals for the $m$/z value corresponding to this formula, bridged by a higher baseline due to tailing (Fig. 4 top panel). The first candidate selected for confirmation of the possible identity of the unknown was 3-oxo-N-phenylbutanamide, since it could easily be acquired from a reputable supplier. However, the retention time of this standard did not match with the retention time observed in the wastewater sample. For the second candidate 3-oxo-2-phenylbutanamide (also known as APAA) it was not possible to obtain a standard from a reputable supplier. Therefore the aqueous solution of the solid yellow substance was analysed by non-target screening. The extracted ion chromatogram (XIC) at a mass window of 5 ppm at $m/z$ 178.08625 revealed two peaks at 8.70 and 13.06 min which had equivalent fragmentation spectra. A possible explanation could be that APAA, a so called β-ketamide, exists in tautomeric forms [23] (see Fig. 2 S.1). In non-polar or aprotic solvents the keto would favour the enol forms [24] but due to hydrogen bond stabilization, especially in the presence of protic solvents like water, the enol form is present in substantial amounts and is probably causing the second signal to appear. The enol form is less polar and hence more retained on the LC-column. Finally the enol tautomer would also explain the —OH group loss through in-source dissociation. The solution of the yellow solid substance was compared with a standard (kindly supplied by the Dutch National Forensics Institute, NFI) of APAA, the identity of which was confirmed by NMR analysis (Fig. 5), and a match was obtained hence confirming our suspicion. This is the first time that APAA has been observed in wastewater and its identity confirmed with a standard.

Interestingly enough the most intense signal C$_6$H$_{11}$N$_2$O in the wastewater sample was also present in the aqueous solution of the solid substance identified as APAA. It therefore appears that the acid hydrolysis of APAA (used to form the precursor BMK) did not result in a transformation of this contaminant. This suggests that this compound can function as a primary synthesis waste marker in sewage water for the use of APAA as a pre-precuror.

Discharging chemical synthesis waste is a real threat for the normal functioning of small wastewater treatment plants. Besides impacting the functioning of the WWTP, the chemical waste will pose a risk for the quality of the receiving waters. This study demonstrates that non-target screening of wastewater collected from WWTPs believed to be impacted by discharges of chemical waste from illicit drugs production can confirm the direct discharge of such waste as well as reveal the actual synthesis process used for the manufacturing of the corresponding drugs. In conclusion, the present study shows that the chemical waste from the illegal manufacturing of stimulants will result in a specific chemical fingerprint that can be tracked in wastewater and used for forensic purposes. To that end WWTP operators should closely monitor any deviations in the physical chemical parameters of the wastewater influent. When a deviation occurs an immediate response monitoring of those deviating parameters should be initiated and samples should be collected in parallel for chemical fingerprinting to allow backtracking.

**Conflict of interest**

The authors declare no conflict of interest.

**Acknowledgments**

We would like to acknowledge Dr. J.D.J. van den Berg from the Netherlands Forensic Institute for supplying the APAA reference standard. We are grateful to M.A. de Boer MSc, van’t Hof Institute for Molecular Sciences of the University of Amsterdam for conducting the NMR analysis of APAA. We are grateful for the cooperation with the water board “Brabantsche Delta” especially J. Oosthoek. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jforsci.2018.03.019.

**References**


