METODE HPLC-DAD UNTUK ANALISIS GLUKOSAMIN PADA TERIPANG KERING DENGAN DERIVATISASI PREKOLOM

(HPLC-DAD Method for Glucosamine Analysis in Dried Sea Cucumber with Precolumn Derivatization)

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ABSTRAK. Glukosamin dikenal sebagai suplemen untuk mengobati osteoartritis dan nyeri sendi. Baru-baru ini masyarakat pesisir Indonesia telah mengembangkan teripang yang diperkaya glukosamin sebagai alternatif pengganti krustasea yang merupakan sumber glukosamin yang banyak dikonsumsi. Tujuan dari penelitian ini adalah melakukan pengembangan dan validasi metode high performance liquid chromatography dengan diode array detector (HPLC-DAD) untuk penentuan glukosamin pada produk teripang. Glukosamin diekstraksi dari sampel teripang kering melalui hidrolisis menggunakan asam klorida 5 M, dilanjutkan dengan derivatifikasi menggunakan larutan Fmoc-Cl (9-Fluorenylmethyl chloroformate) dan buffer borat sebelum dilakukan analisis menggunakan HPLC-DAD. Semua langkah preparasi sampel dan standar dilakukan dengan menggunakan metode pengenceran gravimetri. Pemisahan kromatografi fase terbalik dilakukan di kolom C18 dengan menerapkan elusi gradien menggunakan air dan asetonitril sebagai fasa gerak. Hasil penelitian menunjukkan linieritas yang baik dengan koefisien determinasi sebesar 0,999 pada rentang konsentrasi 1 sampai 220 mg/kg. Metode ini memiliki LOD dan LOQ masing-masing 0,16 dan 0,53 mg/kg, sedangkan nilai perolehan kembali berada di kisaran 98-99%. Nilai presisi intraday ditemukan lebih rendah dari 2% untuk empat konsentrasi spiking yang berbeda (10, 60, 100 dan 200 mg/kg). Metode ini kemudian berhasil diterapkan untuk menganalisis glukosamin pada dua puluh sampel teripang kering lokal. Hasilnya menunjukkan bahwa sebelas sampel mengandung glukosamin dalam rentang 100 hingga 2700 mg/kg.

Kata kunci: fmoc-cl, glukosamin, hplc-dad, teripang

ABSTRACT. Glucosamine is known to be a supplement for treating osteoarthritis and joint pain. Recently, Indonesian coastal communities have developed sea cucumber enriched in glucosamine as an alternative to crustaceans, a widely consumed-glucosamine source. The aim of this study is to develop a high performance liquid chromatography tandem with diode array detector (HPLC-DAD) method for the determination of glucosamine in sea cucumber products was developed and validated. Glucosamine were extracted from dried sea cucumber samples through hydrolysis using hydrochloric acid 5 M, followed by derivatization using Fmoc-Cl (9-Fluorenylmethyl chloroformate) and borate buffer before subjected to HPLC-DAD. All preparation steps for samples and standards were performed using the gravimetric dilution method. The reversed-phase chromatographic separation was conducted in the C18 column by applying a gradient elution of water and acetonitrile. The results showed good linearity with coefficient determination at 0.999 in the range concentration of 1-220 mg/kg. The LOD and LOQ were found to be 0.16 and 0.53 mg/kg respectively, while the recoveries were in the range of 98-99%. The intraday precision values were lower than 2% for four different spiked concentrations (10, 60, 100, and 200 mg/kg). The method then successfully applied to analyze glucosamine in twenty local dried sea cucumber samples. The results showed that eleven of samples contain glucosamine in the range of 100 to 2700 mg/kg.

Keywords: fmoc-cl, glucosamine, hplc-dad, sea cucumber
1. INTRODUCTION

Glucosamine, an amino sugar and a monomer of chitosan, has proven to be effective and widely used for treatment remedies of osteoarthritis (OA) and joint pain due to its chondroprotective effect and anti-inflammatory action (Ibrahim & Jamali, 2010; Lee et al., 2010; Mallu et al., 2010). It is naturally found in human connective tissue and takes part in chitosan and chitin (natural polysaccharide found mainly in exoskeleton of crustaceans) structure (Lopez-Cervantes, Sanchez-Machado, & Delgado-Rosas, 2007; Papich, 2016; Zhu, Cai, Yang, & Su, 2005). Although glucosamine is produced naturally in human body, the amount might not be sufficient to provide a healthy joint muscle, especially for older people. Therefore, the need of glucosamine as a dietary supplement has led to the worldwide consumption of great variety product enriched with glucosamine.

Crustaceans have been widely acknowledged as the primary source of traditional glucosamine. However, Crustaceans are also known to be one of the predominant allergy-inducing foods that can cause anaphylaxis and even death in severe conditions (Davis et al., 2020; Ross et al., 2008; Turner et al., 2017). A number of researches on exploration of other potential glucosamine source from seafood products that have lower potential allergy-inducing have been reported. Sea cucumber has recently gained attention as the potential source of glucosamine, as current studies reported that the glucosamine content in the sea cucumber is found to be around 2% (Bordbar et al., 2011; Khotimchenko, 2018; Rasyid, 2017).

Indonesia is the largest archipelago country in the world, with annual marine resources of 62 million tons. In the last ten years, sea cucumber has become one of Indonesia's marine export commodities due to its high demand, especially in Asia Pacific countries. Indonesia is also known to be the main sea cucumber exporter in the world (Tuwo, 2004). Recently, several food industries and coastal communities in Indonesia have developed dried sea cucumber enriched with glucosamine.

In order to ensure that the products contain enriched glucosamine, an analytical method with sufficient accuracy, sensitivity and selectivity should be developed. Previous studies have reported the glucosamine content in shrimp (Lopez-Cervantes et al., 2007), chitin (Zhu et al., 2005), raw materials (Zhou, Waszkuc, & Mohammed, 2004) and human plasma (Huang et al., 2006; Ibrahim & Jamali, 2010; Song et al., 2012). Several papers have reviewed the nutritional and medicinal benefits of sea cucumber, including glucosamine (Bordbar et al., 2011; Khotimchenko, 2018; Pangestuti & Arifin, 2018). However, the specific study that explore the development method for glucosamine analysis in sea cucumber has yet to be reported. In this study, the analytical method development and optimization for analysis of glucosamine in dried sea cucumber were conducted using high performance liquid chromatography-tandem with diode array detector (HPLC-DAD).

HPLC-DAD was chosen for the analysis due to its low cost procedure with a fast and straightforward method that can be applied in many laboratories. However, as glucosamine lacks of UV-absorbing chromophore, the modification of its structure through derivatization is necessary for the measurement so that the glucosamine can be detected by the diode array detector. Derivatization is a chemical modification process of a compound to produce the new compound which have attributes that are suitable for analysis using GC and HPLC. The chemical structure of the targeted compound remains the same and only the specific functional group of reacting compounds is modified so they can be detectable and analyzable (Zhu et al., 2005).

A number of reagents such as 9-fluorenylmethyl chloroformate (Fmoc-Cl) (Hami et al., 2013; Huang et al., 2006; Zhu et al., 2005), phenylisothiocyanate (PITC) (Du, White, & Eddington, 2004; Tekko, Bonner, & Williams, 2006), 6-aminoquininyl-N-hydroxysuccinimidyl carbamate (AQC) (Wang et al., 2008), and N-(9-fluorenyl-methoxycarboxyloxy) succinimide (Fmoc-Su) (Zhou et al., 2004) have been reportedly used as derivative reagents. Fmoc-Cl was used as a derivative reagent in this study, as many research have proved its effectiveness and stability to form glucosamine-Fmoc adduct, as illustrated in Figure 1 (Zhu et al., 2005). The adduct then can be easily detected by HPLC-DAD (Huang et al., 2006; Li et al., 2013; Lopez-Cervantes et al., 2007; Zhu et al., 2005). However, the reaction using Fmoc-Cl is affected by different parameters such as temperature, pH, buffer solution concentration and organic phase ratio in medium (Mohammadi et al., 2013). Several studies had reported the use of buffer borate with Fmoc-Cl for derivatization reaction at different temperatures with satisfactory results (Catrinck et al., 2014; Hami et al., 2013; Song et al., 2012). In this study,
the derivatization conditions were optimized by varying the pH of the buffer and the ratio of the water-organic phase in the solutions. The optimized method was then applied to the extract of dried sea cucumber samples obtained through acid hydrolysis extraction. Overall, this study covers three parts of method developments: derivatization of glucosamine with Fmoc-Cl and buffer solution, optimization of HPLC-DAD conditions and sample analysis.

Figure 1. Derivatization reaction of glucosamine with Fmoc-Cl.

2. METHOD

Glucosamine hydrochloride crystalline standard (≥99%) and 9-Fluorenylmethyl chloroformate (HPLC grade, 97%, Fmoc-Cl) were obtained from Sigma Aldrich (St. Louis, USA). All reagents were of analytical grade unless otherwise stated and used as received. HPLC grade of acetonitrile and water, acetic acid glacial (≥99%), hydrochloric acid (37%), sodium tetraborate decahydrate, boric acid and sodium hydroxide were purchased from Merck (New Jersey, USA). Water for analytical preparation was produced using Milli-Q Direct 8 water purification system (Merck). Membrane filters 0.45 µm (nylon) and 0.25 µm (PVDF) were purchased from Agilent (Santa Clara, USA).

For chromatography analysis, Agilent Infinity 1260 HPLC-DAD system was used for this study (Santa Clara, USA). This instrument consists of a four channel-degasser, binary pump, automatic liquid sampler (ALS), thermostatted column compartment (TCC) and diode array detector (DAD). The system control, data acquisition and peak integration were performed using Agilent ChemStation B.04.03 software (online and offline mode). Consort C3030 multi-parameter analysis (Turnhout, Belgium) equipped with Mettler Toledo InLab Expert Pro pH electrode (Ohio, USA) was used for pH measurement.

Samples extraction and derivatization

Sea cucumber samples were dried for six hours in 105 °C oven to reduce water content. The dried samples then ground into powder before extraction. Hydrochloric acid with concentrations of 5 M was used in extracting glucosamine from samples. Five grams of samples were hydrolyzed in 50 mL of acid for six hours at 80 °C. The extracts filtered by filter paper before neutralized using NaOH 10 M until pH 7. After that, they filtered by 0.45 µm nylon filter prior to derivatization.

For derivatization, 0.2 M buffer borate solutions with different pH (7, 7.5 and 8) were optimized. The use of buffer is necessary to control the pH of the solution and to stabilize the glucosamine-Fmoc adduct formed during derivatization procedure as the derivatization reaction were affected by different parameters, including the pH of the solution. Moreover, the aqueous based solution facilitates the glucosamine–Fmoc adduct to dissolve in the solution as glucosamine has better solubility in water rather than in organic solvent. After optimization of pH, the amount of buffer added were further optimized from 50 µL to 600 µL.

The stock solution of Fmoc-Cl 5000 mg/kg in acetonitrile was prepared and diluted into 1000 mg/kg. The amount of Fmoc-Cl was also optimized to adjust the ratio of organic phase in the solution during derivatization. Under the optimum condition, 200 µL of samples, 200 µL of buffer solutions and 200 µL of Fmoc-Cl (1000 mg/kg) were added to 1 mL vial and undergo the derivatization reaction. The reaction was conditioned in 40 °C incubator for 30 minutes. The extracts then filtered using 0.25 µm PVDF filter before injected into HPLC system. Gravimetric dilution method was used during both samples extraction and derivatization reaction to calculate the glucosamine content in the samples.
Chromatography and detector conditions

A reversed-phase chromatographic separation was performed on an Agilent Poroshell 120 EC-C18 column (4.6 × 50 mm, 2.7 µm). Water (A) and acetonitrile (B) were used as the mobile phase with both isocratic and gradient elution applied during the optimization. For isocratic elution, 40% A and 60% B were used at a flow rate of 1 mL/min in 10 minutes runtime. While for gradient elution, the same flow rate was applied using the following gradient steps: 0-2 min (90% A), 2-10 min (90-40% A), 10-12 min (40-30% A), 12-14 min (30% A), 14-15 min (30-90% A), resulting in a total runtime of 15 minutes with 2 minutes post time. The column oven temperature was maintained at 30 °C with the autosampler was set at room temperature and 10 µL injection volumes. The 263 nm wavelength was used for analysis with reference wavelength set at 315 nm.

Method Validation

All analytical standards and method validation steps were prepared by the gravimetric dilution method (Kelly, MacDonald, & Guthrie, 2008). Glucosamine standard was dissolved in water to produce a 5000 mg/kg stock solution and diluted into a 1000 mg/kg standard solution. The working standards were made in the range of 0.5 to 220 mg/kg and undergo the same derivatization procedures as samples. The total weight of the solution, including buffer and Fmoc-Cl, was taken into account when calculating the concentration of working standards.

For linearity evaluation, ten concentrations of working standard (1, 5, 10, 20, 40, 60, 80, 100, 150 and 220 mg/kg) were used to construct the calibration curve. The working standards were prepared in three replicates and observed in three different days. For the limit of detection (LOD) and limit of quantitation (LOQ) study, ten replicates of the spiked blank solutions having a concentration of 1 mg/kg were measured. The LOD and LOQ were calculated based on Equation 1 and 2. Where $S_0$ is the estimated standard deviation from the measurement and $n$ is the number of replicate observations (Magnusson & Örnemark, 2014).

\[ \text{LOD} = 3 \times \frac{S_0}{\sqrt{n}} \]  
\[ \text{LOQ} = 3.3 \times \text{LOD} \]

Intraday precision of the instrument was evaluated by the relative standard deviation (%RSD) of two standards concentrations (1 and 30 mg/kg) in ten measurements ($n = 10$). While the accuracy and the precision of the method were evaluated from the recovery of spiked blank solutions in four different concentrations (10, 60, 100 and 200 mg/kg) with three repetitions ($n=3$).

Sample measurement

Twenty samples of dried sea cucumber claimed as enriched in glucosamine were analyzed using the developed method. The samples were supplied from Research and Development Division for Marine Bio Industry, Research Centre for Oceanography, Indonesian Institute of Sciences (LIPI), in the form of dried sea cucumber.

3. RESULT AND DISCUSSION

The optimization of glucosamine derivatization working conditions

During the derivatization process, glucosamine hydrochloride reacts with Fmoc-Cl to form glucosamine-Fmoc, while Fmoc-Cl also reacts spontaneously with water to form Fmoc-alcohol (Fmoc-OH) (Zhang et al., 1996). Fmoc-OH concentration increased significantly over time before finally levelled off during the reaction and inversely proportional to the concentration of Fmoc-Cl. A previous study reported that the rate of reaction increased with an increase in temperature. However, higher temperatures from 60°C to 80 °C decreased the rate of glucosamine derivatization, since it could promote a divergent reaction (Zhu et al., 2005). The AOAC method for determination of glucosamine in dietary supplements suggests that the derivatization performed at 50 °C for 30 minutes (Zhou et al., 2004). In this study, to allow sufficient reaction, the temperature and time of reaction were set at 40 °C and 30 minutes, respectively.

The pH of the reaction were expected to be in the range of neutral pH due to the nature of samples and the glucosamine-Fmoc-Cl reaction (Schneider et al., 2011; Zhou et al., 2004). The reaction was studied in three different near neutral pH (7, 7.5 and 8) with ten repetitions. The peak areas of glucosamine-Fmoc-Cl were observed and the results are shown in Figure 2. Based on the results, the buffer solution with pH 7 gave the highest peak area. However, the repeatability of the measurement of this buffer was relatively low compare to the buffer with pH 8 (Table 1). Since the difference of the average areas between buffer pH 7 and buffer pH 8 was relatively small, buffer pH 8 was chosen for further optimization.
The ratio of water based solution and organic solution in the medium was crucial during the derivatization since two layers medium will inhibit the reaction (Zhu et al., 2005). The effects of these parameters on the peak areas of glucosamine were studied and the results are displayed in Figure 3. The maximum peak area was obtained with a combination of 200 µL of 0.2 M buffer borate pH 8 and 200 µl Fmoc-Cl 1000 mg/kg. The ratio of samples, buffer and Fmoc-Cl at 1:1:1 (v/v) was found to be the optimized ratio for the derivatization. This ratio maintained the concentration of acetonitrile around 33% in the solution and in accordance with the studies that reported Fmoc-Cl was precipitated if the proportion of acetonitrile fell below 30%. Since glucosamine is not soluble in high concentration of organic solvent, the concentration of acetonitrile in the medium should be maintained between 30 and 50 % to ensure that no precipitation occurs during reaction (Huang et al., 2006; Liu et al., 2009).

**HPLC-DAD method optimization**

All dilution steps in this study were performed gravimetrically to reduce the measurement uncertainties. The gravimetric dilution method proves to be more advantageous compared to the volumetric method. It can reduce preparation time, use less solvent consumption and increase the accuracy of the measurement, which makes better reproducibility and improve the quality of analysis (Bhat et al., 2010; Kelly et al., 2008; Melton et al., 1973; Rienitz et al., 2007).

During the development of HPLC condition, water and acetonitrile were used for mobile phase and optimized under different compositions using isocratic and gradient elution. The chromatograms of blank spiked solution at 100 mg/kg in two different elution methods described are exhibited in Figure 4.

As reported by Zhou et al. (2004), glucosamine has two natural stereoisomers (α and β). The interconversion of these two isomers in the solution is inevitable, resulting two peaks of glucosamine in typical HPLC analysis. The sum of the areas from the two peaks is used for the quantification of glucosamine in the samples (Zhou et al., 2004).

Based on Figure 4, both isocratic and gradient elution gave two peaks of glucosamine. However, glucosamine peaks in the isocratic elution gave poor resolution as the peaks were not baseline resolved (Table 3). The gradient elution provides better separation and resolution for the
elution method has proven to improve the quality of the separation. Therefore, the gradient elution method was chosen for method validation and sample analysis. Table 3 shows the result of mobile phase optimization in each elution method, while the optimized HPLC conditions used for validation and sample analysis were listed in Table 2.

Table 2. Optimum conditions for HPLC-DAD analysis.

<table>
<thead>
<tr>
<th>Column</th>
<th>C18, 4.6 x 50 mm, 2.7 µm particle size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase</td>
<td>Water and acetonitrile</td>
</tr>
<tr>
<td>Elution method</td>
<td>Gradient elution</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1 mL/min</td>
</tr>
<tr>
<td>Runtime</td>
<td>15 min</td>
</tr>
<tr>
<td>Injection temp.</td>
<td>Room temperature</td>
</tr>
<tr>
<td>Injection vol.</td>
<td>10 µL</td>
</tr>
<tr>
<td>Column Temp.</td>
<td>30 °C</td>
</tr>
<tr>
<td>Wavelength</td>
<td>263 nm (reference at 315 nm)</td>
</tr>
</tbody>
</table>

**Linearity, LOD and LOQ**

An external multipoint calibration technique was applied for the instrument calibration. The linearity of the detector response was evaluated by a set of standard solutions with concentration ranging from 1 to 220 mg/kg in three different days. The linearity, expressed as coefficient determination (R²), was found to be satisfactory, as R² was 0.999 for all curves (Figure 5) with a typical equation for the curve was y = 43.57C + 12.35. The LOD and LOQ of the instrument were calculated at 0.16 and 0.53 mg/kg, respectively. These LOD and LOQ values are expected from the HPLC-DAD measurement and well below the expected samples concentration, in which the glucosamine content in the products was reported to be in the ppm to percentage level. Table 4 summarizes the linearity, LOD, and LOQ of the developed method.

Table 3. Optimization of mobile phase composition.

<table>
<thead>
<tr>
<th>Elution method</th>
<th>Retention time of glucosamine (minutes)</th>
<th>Number of theoretical plates (N)</th>
<th>HETP (cm)</th>
<th>Asymmetry factor</th>
<th>Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rt₁, Rt₂</td>
<td>Rt₁, Rt₂</td>
<td>Rt₁, Rt₂</td>
<td>Rt₁, Rt₂</td>
<td></td>
</tr>
<tr>
<td>Isocratic</td>
<td>0.426, 0.533</td>
<td>1292, 1046</td>
<td>3.87E-03, 4.78E-03</td>
<td>0.86, 1.14</td>
<td>1.89</td>
</tr>
<tr>
<td>Gradient</td>
<td>7.481, 7.692</td>
<td>361061, 414326</td>
<td>1.38E-05, 1.21E-05</td>
<td>0.92, 0.96</td>
<td>4.32</td>
</tr>
</tbody>
</table>
The precision and accuracy of the method were calculated from the spiked blank solution at different concentrations. The concentration then calculated by an external multi-point calibration technique using the observed peak areas. The accuracy, expressed as a percentage, was determined as the difference between the calculated values obtained from the calibration curves and the nominal values from gravimetric dilution. The accuracy and intra-day precision of the method are summarized in Table 5.

### Table 5. Intra-day precision and accuracy of glucosamine analysis

<table>
<thead>
<tr>
<th>Spiked Concentration (mg/kg)</th>
<th>Assay Concentration (mg/kg)</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.0</td>
<td>11.8</td>
<td>98.7</td>
<td>1.8</td>
</tr>
<tr>
<td>64.8</td>
<td>64.7</td>
<td>99.7</td>
<td>0.7</td>
</tr>
<tr>
<td>99.1</td>
<td>99.0</td>
<td>99.8</td>
<td>0.9</td>
</tr>
<tr>
<td>218.2</td>
<td>214.8</td>
<td>98.5</td>
<td>1.7</td>
</tr>
</tbody>
</table>

These results meet the criteria of AOAC Appendix F: Guidelines for Standard Method Performance Requirements, which stated that the recommended acceptable recovery for concentration in the range of 10-100 ppm is 90-107%. The RSD value for all concentrations was also meet the criteria as it was found to be smaller than 5.3% (AOAC, 2016).

### Sample analysis

The optimized HPLC method was applied for determining the glucosamine content in twenty samples of dried sea cucumber. The samples were supplied from local marine industry in the dried form. Two repetitions of each sample were prepared and based on the results, glucosamine was detected in eleven samples, as displayed in Figure 6.

![Figure 6. Mean of glucosamine contents in dried sea cucumbers](image)
The results indicated that the content of glucosamine in the samples was varied from around 100 to 2700 mg/kg. Previous study reported the glucosamine content in sea cucumber using derivatization method was found to be less than 5000 mg/kg (Pringgenies, Rudiyanti, & Yudiati, 2018) and could be as high as 20000 mg/kg (Rasyid, 2017). This variation may happen due to the difference in industrial processes, such as the source of sea cucumber used, production process, and preparation conditions. The standard deviation of each sample measurement is listed in Table 6.

### Table 6. Standard deviation for each sample measurement.

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Glucosamine content (mg/kg)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1550</td>
<td>7.6</td>
</tr>
<tr>
<td>3</td>
<td>2248</td>
<td>2.7</td>
</tr>
<tr>
<td>4</td>
<td>530</td>
<td>6.1</td>
</tr>
<tr>
<td>6</td>
<td>1493</td>
<td>6.5</td>
</tr>
<tr>
<td>8</td>
<td>1189</td>
<td>6.1</td>
</tr>
<tr>
<td>10</td>
<td>1411</td>
<td>5.8</td>
</tr>
<tr>
<td>12</td>
<td>1789</td>
<td>2.8</td>
</tr>
<tr>
<td>16</td>
<td>2741</td>
<td>3.0</td>
</tr>
<tr>
<td>17</td>
<td>92</td>
<td>3.1</td>
</tr>
<tr>
<td>19</td>
<td>2571</td>
<td>2.2</td>
</tr>
<tr>
<td>20</td>
<td>1841</td>
<td>7.2</td>
</tr>
</tbody>
</table>

### 4. CONCLUSION

A new HPLC-DAD method for the determination of glucosamine in dried sea cucumber was successfully developed and validated. Precolumn derivatization of standards and samples was conducted using Fmoc-Cl as derivatizing reagent. The derivatization conditions were also optimized. The chromatographic separation was performed in the C18 column under gradient elution of water and acetonitrile as the mobile phase. The validation parameters such as selectivity, linearity, LOD, LOQ, accuracy, and precision were evaluated with satisfactory results. The developed method was then applied to analyze the sea-cucumber enriched in glucosamine. The results showed that from twenty samples, eleven of them contained glucosamine in the range of 100 – 2700 mg/kg. The method presented in this study offers simple and inexpensive procedures with less consuming reagents, which can be proposed as an alternative method for routine laboratory analysis.

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