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IMPROVED ANALYTICAL METHOD OF SYNTHETIC FOOD COLOUR ADDITIVE, BROWN HT BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract

Brown HT is bis-azo dye which is permitted in EU as a color additive. C18 column was generally applied in analysis of Brown HT in HPLC system using diode array detection with sodium sulfate or phosphate solution as an eluent. C4 column can also be used with 100% methanol as mobile phase. Low recovery, reproducibility, and linearity are the major limitations in previous reported methods. The purpose of this study is to optimize the method using different solvents. In our study, the best resolution of Brown HT analysis was observed with following eluent; solvent A of mobile phase was 0.575g mono ammonium phosphate, 0.7g disodium hydrogen phosphate in 500mL water added with 500mL methanol (pH 6.9) and solvent B was methanol. This method exhibited quite high recovery and reproducibility of Brown HT and it presented better result compared to the previous studies. LC-MS/MS was also used to confirm Brown HT.

Key words: *Colour Additive, Food Additive, High-performance liquid chromatography (HPLC), LC-MS/MS, Brown HT, CI Food Brown 3, E155*

1. INTRODUCTION

Brown HT (E155) is a synthetic bis-azo dye food colouring consisting of reddish-brown powder or granules with molecular formula of $C_{27}H_{18}N_4Na_2O_9S_2$. It has a molecular weight of 652.56 g/mol with CAS Registry Number of 4553-89-3. Disodium 4,4'-(2,4-dihydroxy-5-hydroxymethyl-l,3-phenylenebisazo)di-(naphthalene-l-sulfonate) is well known for its full chemical name (Directive 94/36/EC; Directive 95/45/EC). Its structural formula is given in Figure 1. There are at least 10 synonyms for this compound (ChemIDplus advanced, 2007). The most commonly used synonyms in published literature are Brown HT, Chocolate Brown HT and CI Food Brown 3. It is soluble in water, but insoluble in ethanol. It presents very good stability in light and heat (Leo M.L. Nollet, 2012).

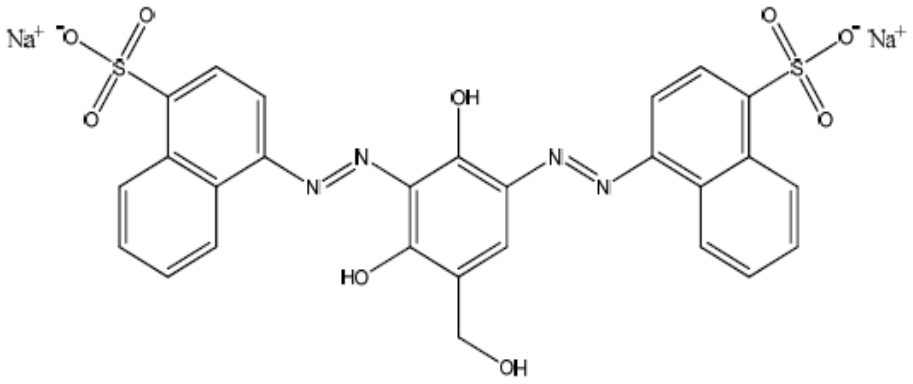


Fig. 1: Structural formula of Brown HT.

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Brown HT consists essentially of disodium 4,4'-(2,4-dihydroxy-5-hydroxymethyl-l,3-phenylenebisazo) di-(naphthalene-l-sulfonate) and subsidiary colouring matters, together with sodium sulphate and/or sodium chloride as the principal uncoloured components. The sodium salt is permitted in Brown HT as well as the potassium salts and calcium (Commission Directive, 2008).

The purity is specified as not less than 70% of total colouring matters, calculated as the sodium salts of other salts which remain as 30% of calcium or sodium chloride or calcium or sodium sulphate (but this is never mentioned in detail), ≤ 10% subsidiary colouring matters, ≤ 0.01% unsulphonated primary aromatic amines, ≤ 0.7% 4-aminonaphthalene-1-sulphonic acid and ≤ 0.2% ether extractable matter, depending on the manufacturing process. (Commission Directive, 1995)

The United States do not permit Brown HT and it is not approved by WHO. However, this colour additive was approved in food by the EU as follows; nonalcoholic flavored drinks, red fruit preserves, candied fruits and vegetables, confectionery and bakery products, decorations and coatings, edible ices, cheese desserts including flavored milk products, seasonings, sauces, relishes chutneys, pickles, and piccalillis, mustard, fish and crustacean pastes, fish roe, nutritional supplement, snacks, liquid and solid food supplement integrators, soups, meat/fish analogs based on vegetable proteins, and spirit beverages (FAO/WHO, 2006; Pintea, 2008; US Food & Drug Administration, 1999).

Brown HT is firstly evaluated for food application by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1977 and followed by the EU Scientific Committee for Food (SCF) in 1984. In 1984 JECFA set an Acceptable Daily Intake (ADI) of 0-1.5 mg/kg bw/day, while the SCF reported an ADI of 0-3 mg/kg bw/day (EFSA, 2010).

It exhibited compound-related but not dose-related toxicity in a short-term toxicity studies with rats. Dose-dependent toxicity was observed only in condition that would not lead to a lowering of the current ADI (Chambers PL, 1966).

Bacterial genotoxicity tests have been performed with Brown HT, but only negative results were reported. The activation process of these bis-azo dyes in animals is complex and bacterial tests with S9 might not be suitable to detect mammalian genotoxicity in general (Haveland-Smith RB, 1980).

The specifications on the purity of Brown HT explains that the concentrations of unidentified unsulphonated aromatic amines can be present in concentrations of up to 100mg/kg Brown HT. Although it is possible that the aromatic amines are related to genotoxicity or even carcinogenicity, Brown HT exhibited negative in *in vitro* genotoxicity as well as in long-term carcinogenicity studies. (Jung R, 1992).

Long term toxicity and carcinogenicity studies with Brown HT were studied with rats and mice. No carcinogenic effects were reported in either species. No adverse effects were observed in rats at dietary dose levels up to 425 mg/kg bw/day (highest dose tested). However, several effects were found in the long-term mouse study at the highest dose tested (715mg 85% pure Brown HT/kg bw/day). The NOAEL in the mouse study was 0.1% in the diet equivalent to 143mg Brown HT/kg bw/day. (Carpanini F-MB, 1978)

In previous reports, JECFA reviews studies about Brown HT in rats (2 studies) and pigs (1 study). If rats were administered 0, 0.5, 1.0, or 2.0% in the diet for 21 weeks, no effect was observed on appearance or condition. Without decreased food intake, growth was retarded at 1 and 2%. No effect was revealed on haematological parameters. A mild renal dysfunction was occurred at 1 and 2%. Increased relative weight of brain, adrenals, spleen, kidneys and ovaries and brown pigmentation of liver Kupffer cells, kidneys, and lymph nodes were major symptoms. Growth retardation was found on 1 and 2% in diet for 90 days and appearance, behavior, or survival rate did not changed. Decreased haemoglobin, red cell count and haematocrit were measured at 2% but, pathological damage was observed. Pigmentation of intestinal cells, lymph nodes, and in kidneys was interesting characteristics at 2%. When pigs were administered with Brown HT (0, 5, 20, or 100 mg/kg bw/day) for 13 weeks (route not specified), no adverse effect were found on mortality, growth, organ weight, and urine composition. However, the haemoglobin concentration was reduced in male pig at all three levels. No histopathological results were reported. (FAS 12-JECFA21, 1977)

So far, several analysis methods with HPLC system for Brown HT were developed. However, very low reproducibility and recovery were still drawbacks of these methods using sodium sulphate solution. To optimize analysis and detection of this food additive, various elution conditions were investigated using different column and eluents with HPLC system and improved recovery, reproducibility and linearity will be presented in this study.

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2. MATERIAL AND METHODS

2.1. Materials

Water, methanol (MeOH) and Acetonitrile (MeCN) were purchased from Burdick & Jackson (USA). Sodium sulphate (anhydrous, 98.5%) and sodium phosphate dibasic (anhydrous, 99.0%) were products of SAMCHUN CHEMICALS (Pyeongtaek-city, Korea). Phosphoric acid and ammonium phosphate monobasic were obtained from DUKSAN PURE CHEMICAL CO., LTD. (Ansan-city, Korea). Brown HT was supplied from FastColours LLP (Scissett, England). It was dissolved in water and another one was dissolved in mixture of methanol : water added with mono ammonium phosphate, sodium phosphate dibasic, methanol and phosphoric acid (50:50).

2.2. Instruments and chromatography

HPLC system includes 305 pump, 306 pump, 811C dynamic mixer, UV/VIS-151 (detection of Brown HT was 460nm) and 234 auto injector from Gilson was applied for the analysis of Brown HT. Eclipse XDB-C18 column (4.6 x 150mm x 5µm, from Agilent, USA) were used to separate Brown HT and Viva C4 column (4.6 x 150mm x 5µm, from Restek, USA) was equipped when methanol was used as a eluent.

2.3. Methods

2.3.1. Analysis of Brown HT with C18 column using sodium sulfate solution

Solvent A of mobile phase was 0.1M Sodium sulphate, pH 2.5 with phosphoric acid and solvent B was solvent A:water:methanol (1.5:7:22, v/v). Gradient was 0-100% solvent B in 20min, 100% solvent B for 10min. Injection volume was 10µl and flow rate was 1.5 ml/min. One thousand µg of sample was dissolved in 1ml of water (Leo M.L. Nollet, 2000).

2.3.2. Analysis of Brown HT with C4 column using methanol

One hundred percent methanol was used as the mobile phase with C4 column. Injection volume was 5µl and flow rate was 1 ml/min. Sample of 1000µg was dissolved in 1ml of water (N.P.Boley, 1980). Decreased methanol concentration was applied as a mobile phase to increase the resolution at same elution condition at 70% instead of 100% methanol in next step.

2.3.3. Analysis of Brown HT with C18 column using phosphate solution

Solvent A of mobile phase contained 0.575g mono ammonium phosphate, 0.7g disodium hydrogen phosphate in 900mL water with 100mL methanol. pH was adjusted using phosphoric acid to 6.9 and solvent B was methanol. Gradient conditions were explained in Table 1. One thousand µg of sample was dissolved in 1 ml of solvent A. Injection volume was 1µl and flow rate was 0.8 ml/min (Minjia HUANG, 2011). HPLC analysis was performed at 40℃.

In order to increase resolution, solvent A was prepared in 50% methanol instead of 90% methanol at same elution condition except column temperature (room temperature). Gradient steps were explained in Table 2 and Brown HT was dissolved in this solution at the concentration of 1000µg per one ml.

2.3.4. Analysis of Brown HT using LC-MS/MS

LTQ-Velos (Thermo Scientific, USA) LC-MS/MS system was used for further identification of Brown HT. Solvent A was acetonitrile (MeCN) containing formic acid (0.1%) and solvent B was water with formic acid (0.1%) . Purified Brown HT was directly injected to MS/MS for the mass spectrometry analysis.

Time (min)	B%
0	14
4.5	29
8	29
9	50
10.5	65
12	90
14	100
15	14

Table 1: Gradient condition with phosphate solution : methanol (9:1, v/v).

Time (min)	B%
0	14
4.5	29
8	29
9	50
10.5	65
20.5	90
22	100
24	14

Table 2: Gradient condition with phosphate solution : methanol (5:5, v/v).

3. RESULT AND DISCUSSION

3.1. HPLC analysis with C18 column using sodium sulfate solution

Figure 2 presents HPLC analysis of Brown HT with C18 column (Leo M.L. Nollet, 2000). As shown in chromatogram, at the end of gradient tiny peaks were detected at very low level of detectin and most Brown HT was disspeared during separation even though 10µl of sample was injected. In addition, low reproducibility was still in problem. A lack of reproducibility is a clear indication of a drawback of this method. Furthermore, the intensity of detected peaks was too low at 460nm. From these results, this method with C18 column using sodium sulfate solution was proved as not suitable in analysis method for Brown HT.

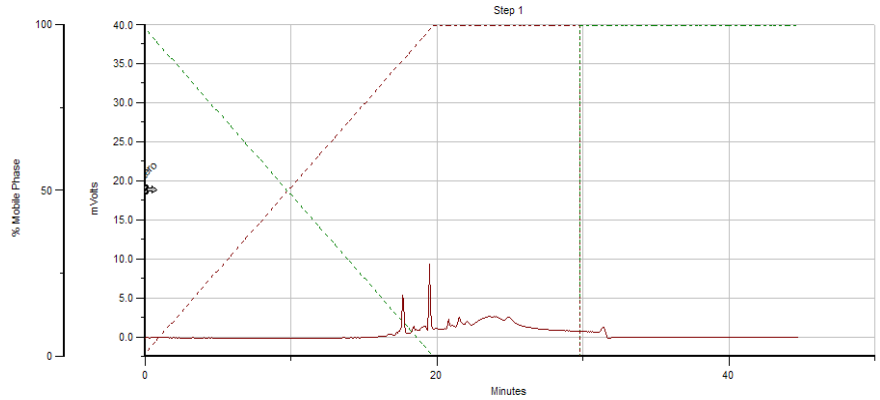


Fig. 2: HPLC chromatogram of Brown HT with C18 column using sodium sulfate solution at 460nm. Red and green dotted lines were concentration of solvent B and A, respectively.

3.2. HPLC analysis with C4 column using methanol as a eluent

Figure 3 presents HPLC chromatogram of Brown HT with C4 column using 100% methanol as the mobile phase (N.P.Boley, 1980). Strong intensity of the detection was observed at the beginning of the elution at 1.36 min of retention time. The intensity of the peaks depended on the concentration of Brown HT dose dependently and it showed high reproducibility. The major drawbacks of this method are the lowest resolution and too fast retention time. Brown HT could not be separated enough from other contaminants in the sample. Therefore, the concentration of the methanol was decreased to 70% as mobile phase to improve the resolution. As shown in

Figure 4, the retention time of peak appeared to be at 3.01min after injection, which is slightly retarded than before. The peak intensity depends on the injected sample amount.

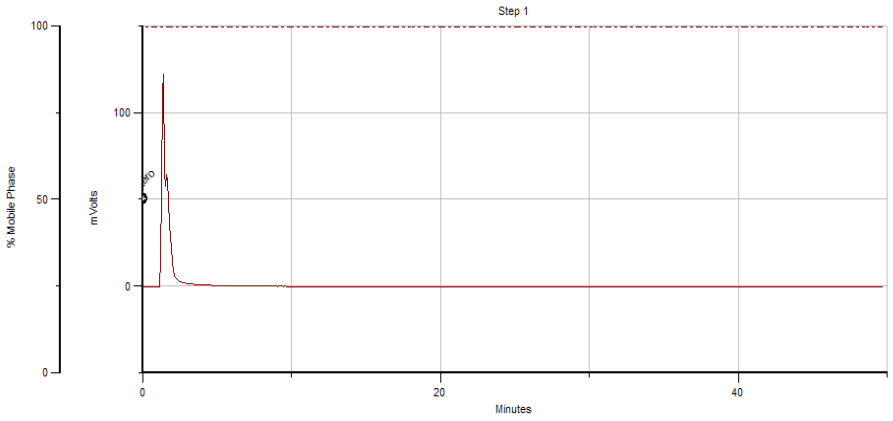


Fig. 3: HPLC analysis of Brown HT with C4 column using 100% methanol as an eluent.

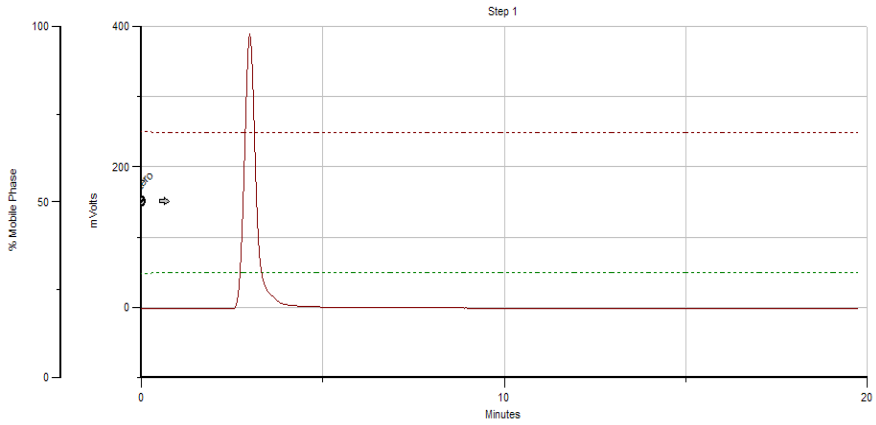


Fig. 4: HPLC chromatogram of Brown HT with 70% methanol as an eluent at 460nm. Red dotted line is pump of methanol and green dotted line is pump of water.

3.3. HPLC analysis with C18 column using phosphate solution

HPLC analysis of Brown HT using phosphate solution with 10% of methanol (9:1, v/v) was presented in Figure 5 (Minjia HUANG, 2011). As shown in chromatogram, resolution of the peaks separation was low and Brown HT appeared at the end of agglomerated peaks as a highest peak. Furthermore, the reproducibility of the analysis was very low. If the level of methanol increased to 50% instead of 10%, the resolution increased very much as shown in Figure 6 and many peaks could be observed during elution. Major peak, Brown HT appeared at the end of separation, 12.5 min after injection. This method exhibited relatively high recovery and reproducibility compared with other methods. Therefore, HPLC analysis with C18 column using phosphate solution with 50% methanol has been proved as an appropriate analysis method so far.

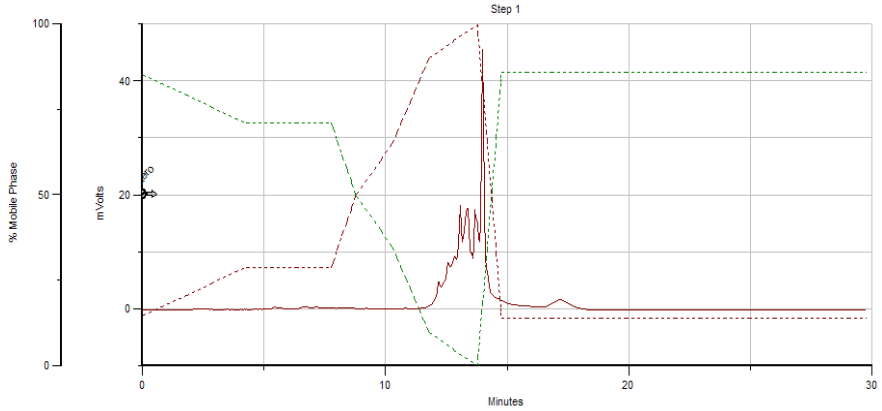


Fig. 5: HPLC chromatogram of Brown HT with C18 column using phosphate solution : methanol (9:1, v/v) at 460nm. Red and green dotted lines were concentration of methanol and phosphate, respectively.

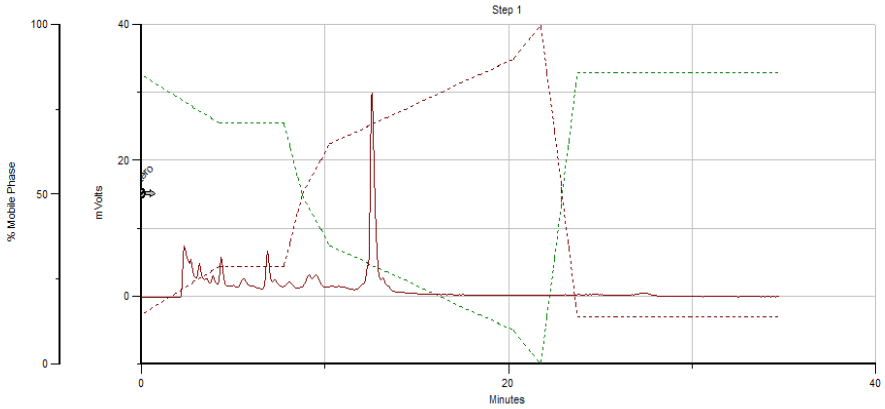
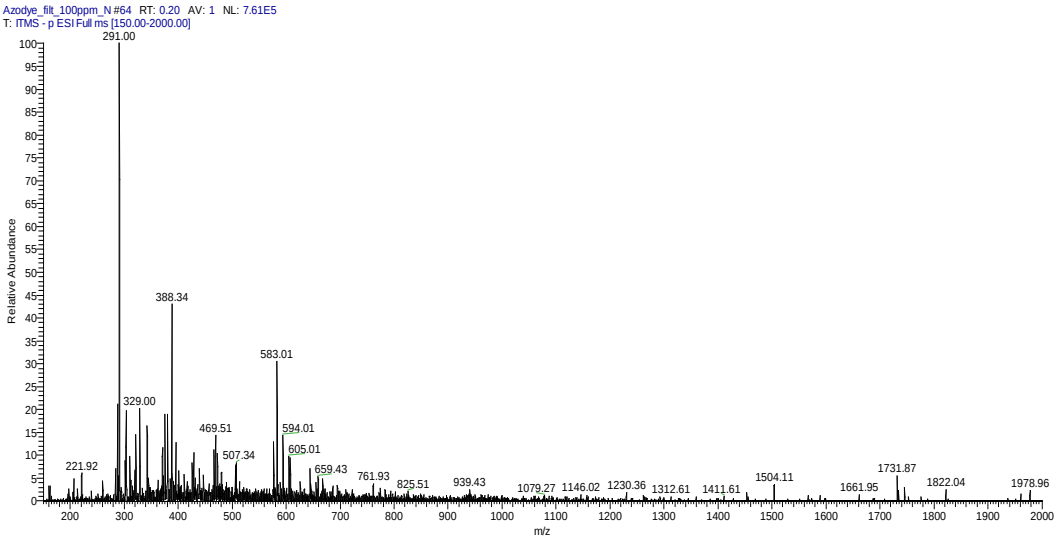


Fig. 6: *HPLC chromatogram of Brown HT with C18 column using* phosphate solution : methanol (5:5, v/v) at 460nm. Red and green dotted lines were concentration of methanol and phosphate, respectively.

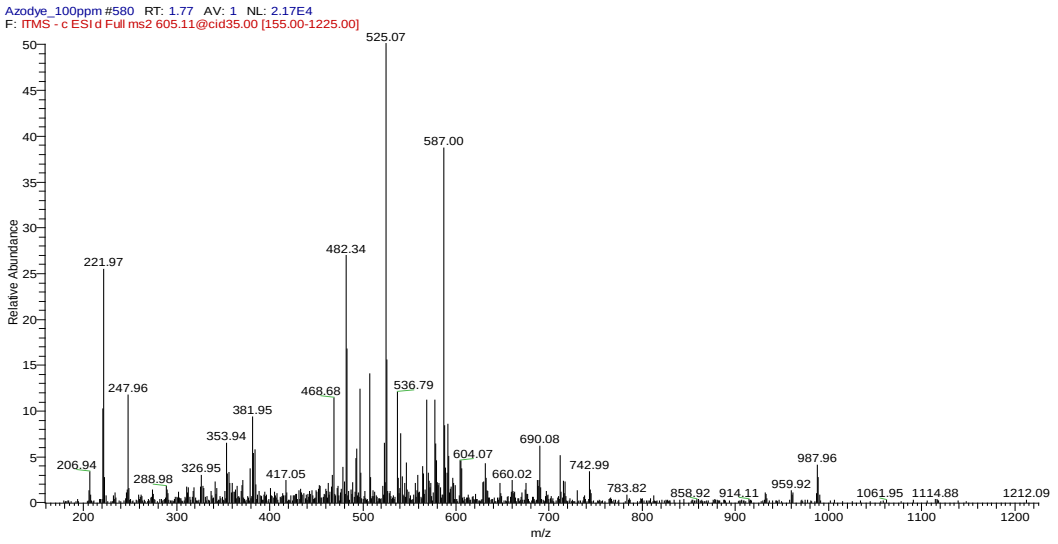
3.4. Result of analysis with LC-MS/MS

Full mass scan of Brown HT was shown in Figure 7-a. A peak m/z value of 605 can be explained as a mother backbone of Brown HT except two sodium ions. This peak was analyzed in MS/MS and following peaks with m/z value of 525 which lost one SO3 ion could be found as the highest peak (Figure 7-b). Interestingly, three peaks with m/z values of 291.00, 388.34 and 583.309 were found as major peak in full mass chromatogram. The highest peak with m/z value of 291.00 could be postulated as azo-backbone with sodium sulfate with hydroxyl group and formic acid. The peak with m/z value of 583.09 is guessed as a couple of molecular with size 291.00. Further studies will be performed to understand the patterns of ionization of Brown HT in LC-MS/MS

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(a)



(b)

Fig. 7: Full mass scan scan of Brown HT using LC-MS/MS (a) and MS/MS chromatogram of a peak with m/z value of 605 (b).

4. CONCLUSIONS

HPLC analysis of Brown HT using phosphate solution with 50% of methanol (5:5, v/v) has been proved as an appropriate analysis method so far. Solvent A of mobile phase contained 0.575g mono ammonium phosphate, 0.7g disodium hydrogen phosphate in 500mL water with 500mL methanol. The resolution of Brown increased and this method exhibited relatively high recovery and reproducibility compared with other methods. A peak m/z value of 605 full mass scan of Brown HT can be explained as a mother backbone and 525 which lost one SO3 ion could be found as the highest peak in MS/MS analysis. Further studies will be required to understand the patterns of ionization of Brown HT in LC-MS/MS.

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