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STUDY OF STEVIA EXTRACTION CONDITION TO GET THE HIGHEST STEVIOL GLYCOSIDE CONTENT USING HPLC-ELSD METHOD

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ABSTRACT

Stevia contains steviol glycosides which not only have sweet taste but also have anti-diabetic activity. Several main steviol glycoside constituents namely stevioside, rebaudioside A, rebaudioside C and dulkoside are responsible for stevia sweet taste. People with diabetes mellitus are suggested not to include sugar (sucrose) in their meals and drinks and preferably using sugar free sweeteners. Stevia which obtained from Stevia rebaudiana plant is a safe and popular sugar free sweetener. The chemical structure of steviol glycosides (stevioside, rebaudioside A, rebaudioside C and dulcoside A) are very similar causing separation rather difficult moreover their structures do not have chromophore group thus, their UV absorption are very low. Since analysis of these compounds using HPLC with UV detector giving undesirable result, it is necessary to use a detector that is not dependent on a chromophore group, such as an ELSD detector. Analysis of steviol glycoside content in stevia from various extraction methods (soxhletation, percolation and maceration) and solvents (ethanol and methanol) were done by using HPLC-ELSD. Largest Steviol glycoside contents were found in ethanol extract from maceration method.

KEYWORDS: Extraction, Stevia, stevioside, rebaudioside A, rebaudioside C and dulcoside A HPLC-ELSD.

INTRODUCTION

The main steviol glycosides in stevia are steviosides, rebaudiosides A, rebaudiosides C and dulcosides A. Steviol glycosides is known to have anti-diabetic properties. Diabetic population in Indonesia has been increasing every year, becoming the world 5th largest country with diabetic population, estimated to be about 10% of the population and about 51% of those were undiagnosed (AFES, 2017). People with diabetes mellitus are suggested not to include sugar (sucrose) in their meals and drinks and recommended to use safe sugar free sweeteners. Sugar free natural sweeteners which is safe for diabetes mellitus patients is stevia from the Stevia rebaudiana Bertoni plant (Supriyadi et al., 2016). Stevia rebaudiana is also traditionally used to treat diabetes mellitus. The use of Stevia rebaudiana leaves as a substitute for sugar does not raise blood sugar levels and does not cause obesity (Brahmachari et al., 2011). The use of stevia sweeteners can also reduce weight (Elnaga et al., 2016).

The sweet taste of Stevia rebaudiana is due to the presence of several steviol glycoside compounds, including stevioside (± 13,955%), rebaudioside A (± 1%), rebaudioside C (± 0.5%), and dulcoside A (± 0.56%) (Supriyadi et al., 2016) Stevia has a sweet taste 30 times sweeter than sugar (sucrose), while pure stevioside has a sweet taste 200 times sweeter than sucrose (Raini and Isnawati, 2011).

According to WHO (2006) Acceptable Daily Intake (ADI) for steviol glycosides from Stevia rebaudiana is 4 mg / kg / day. The maximum recommended dose regulated in Japan is 3 mg / kg / day and in America 5 mg / kg / day. At this dose, stevia is safe to consume as a sugar substitute sweetener without giving calorie (Shanon, 2016). According to the Food Drug and Administration (FDA) (2008), Stevia is a safe product for consumption of up to 1500 mg per day, but the FDA does not recommend stevia to be used for pregnant and lactating women. WHO (2006) concluded that steviosides and rebaudiosides were not mutagenic both in vitro and in vivo. WHO also reports that steviosides, rebaudiosides and steviol are not carcinogenic. Stevioside also provides pharmacological effects on patients as antihypertensive and antidiabetic (Benford et al., 2006). Steviosides are stable on high temperatures, similar to saccharin and aspartame, the compound is resistant to heating up to 200oC, so it can be used as a sweetener in almost all types of food (Raini and Isnawati, 2011).

Stevia products are generally obtained from Stevia rebaudiana plants through extraction and purification (Purkayastha et al., 2016).

The use of Stevia rebaudiana extract in Indonesia has been permitted by the Food and Drug Supervisory Agency (BPOM) with the Decree of the Head of POM No. HK.00.05.52.3877 of 2004 and has been renewed with the Decree of the Head of the National Agency of Drug and Food Control No. 4 of 2014, concerning the Requirements for the Use of Stevia Extracts as Natural Sweeteners. Stevia product quality assurance is an important aspect to guarantee the quality of products circulating in the market and also in the effort to fulfill consumer protection laws. Quality assurance aims to ensure that the product is safe to use by consumers and has a composition in accordance with product label.

Steviol glycosides in Stevia rebaudiana are mainly steviosides, rebaudiosides A, rebaudiosides C and dulcosides A. If the product mention stevia or stevia sweetener on its label then the product must contain stevioside, rebaudioside A, rebaudioside C and dulcoside A, if not the product can be said to be a counterfeit. Currently many products labeled as stevia or stevia sweetener circulating on the market, it is necessary to detect the content of stevioside, rebaudioside A, rebaudioside C and dulcoside A in these products. Detection of counterfeit stevia products was done using a Raman spectometer and the results obtained were some samples of stevia products did not contain stevia sweeteners (Jentzsch et al., 2016). Detection of counterfeit stevia products using HPLC-ELSD was done by comparing HPLC-ELSD chromatograms from extracts of stevia products with HPLC-ELSD chromatogram of standard mixture solutions of stevioside, rebaudioside A, rebaudioside C and dulcoside A (Supriyadi et al., 2018).

MATERIAL AND METHOD

Dry stevia leaves (from Tawangmangu), Standard Steviosida, Rebaudioside A, Rebaudioside C and Dulcoside A from Sigma-Aldrich. Methanol and acetonitrile from E-Merck and distilled water. Extractions of steviol glycoside content from stevia were carried out by socletation, percolation and maceration with stirring and boiling of the solvent. For the analysis of steviol glycoside content was done by using HPLC-ELSD.

The analysis of a mixture of Steviosida, Rebaudioside A, Rebaudioside C and Dulkoside A was done by using Luna Phenomenex column with acetonitrile water = 35: 65 flow rate of 1 ml / minute, column temperature 50^{o}C, temperature nebulation and evaporation temperature detector 40 and 50^{o}C (Supriyadi et al., 2016). A standard solution mixture of Steviosida, Rebaudioside A, Rebaudioside C and Dulcoside A solutions was injected into HPLC-ELSD at optimum conditions obtained by the chromatogram. Stevia extract samples from several solvents and extraction methods were also injected into HPLC-ELSD, the obtained chromatograms then were compared with standard solution chromatograms to determine levels of steviol glycosides in sampel solution.

RESULT AND DISCUSSION

Standard solutions of stevioside, rebaudioside A, rebaudioside C and dulcoside A was injected into HPLC-ELSD. Chromatogram standard solution of stevioside, rebaudioside A, rebaudioside C and dulcoside A with Luna Phenomenex 100A column (250 x 4.5 mm) mobile phase of acetonitrile: water (35: 65) had given desired separation

As shown in Figure 1, Chromatogram had fulfilled the optimum condition criteria, the chromatogram separation (Rs) was more than 1.5, the Rs of first and second chromatograms was 1.86, the second and third chromatograms was 6.17 and the third and fourth chromatograms was 2.47.

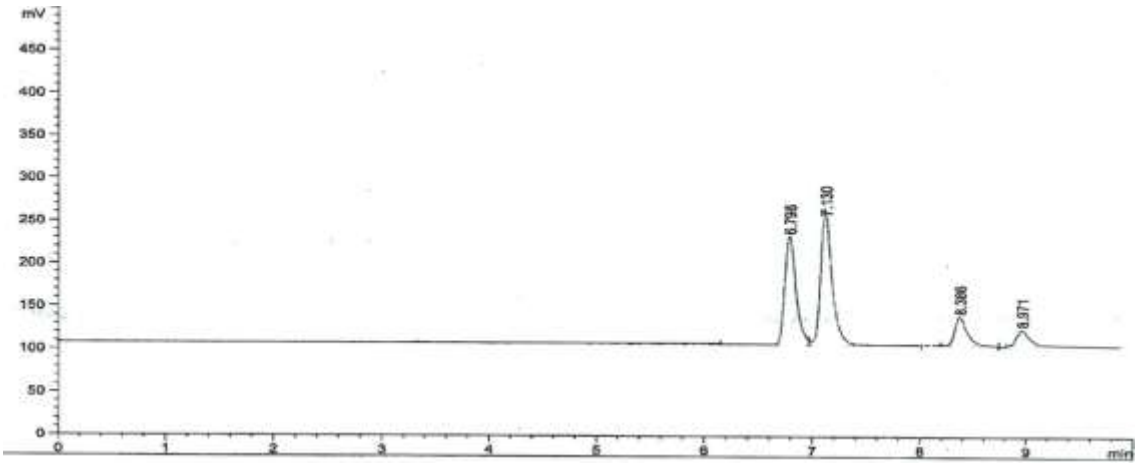

Fig. 1: Chromatogram standard solutions mixture of stevioside, rebaudioside A, rebaudioside C and dulcoside A with Luna column Phenomenex mobile phase of acetonitrile: water (35: 65) flow rate of 1 ml / minute.

To determine the concentration of stevioside, rebaudioside A, rebaudioside C and dulcoside A in sample solution. Previously we determined calibration curves and linear regression equations of standards (stevioside, rebaudioside A, rebaudioside C and dulcoside A) based on peak area of the chromatogram versus their concentration.

For standard stevioside solutions as follows

Steviosida (ppm)	Luas puncak (mV)
8,6	52,79
17,2	132,03
25,8	228,07
34,4	312,85
43	406,64

y = 10,332x - 40,08 dan R = 0,999

For standard rebaudiosida-A solutions as follows

Rebaudiosida A (ppm)	Luas puncak (mV)
20,2	191,38
30,3	339,68
40,4	497,13
50,5	654,07
60,6	765,04

y = 14,47x - 95,22 dan R = 0,999

For standard rebaudiosida-C solutions as follows

Rebaudiosida C (ppm)	Luas puncak (mV)
20,2	52,8
30,3	95,1
40,4	145,47
50,5	202,53
60,6	254,34

y = 5,054x - 54,15 dan R = 0,999

For standard Dulkosida-A solutions as follows

Dulkosida A (ppm)	Luas puncak (mV)
18,6	54,29
27,9	94,69
37,2	139,77
46,5	179,25
58,8	230,09

y = 4,399x - 26, 68 dan R = 0,999

Analysis of stevia extract obtained by using soxhletation with ethanol solvent for seven circulation, HPLC-ELSD obtained the chromatogram as follows:

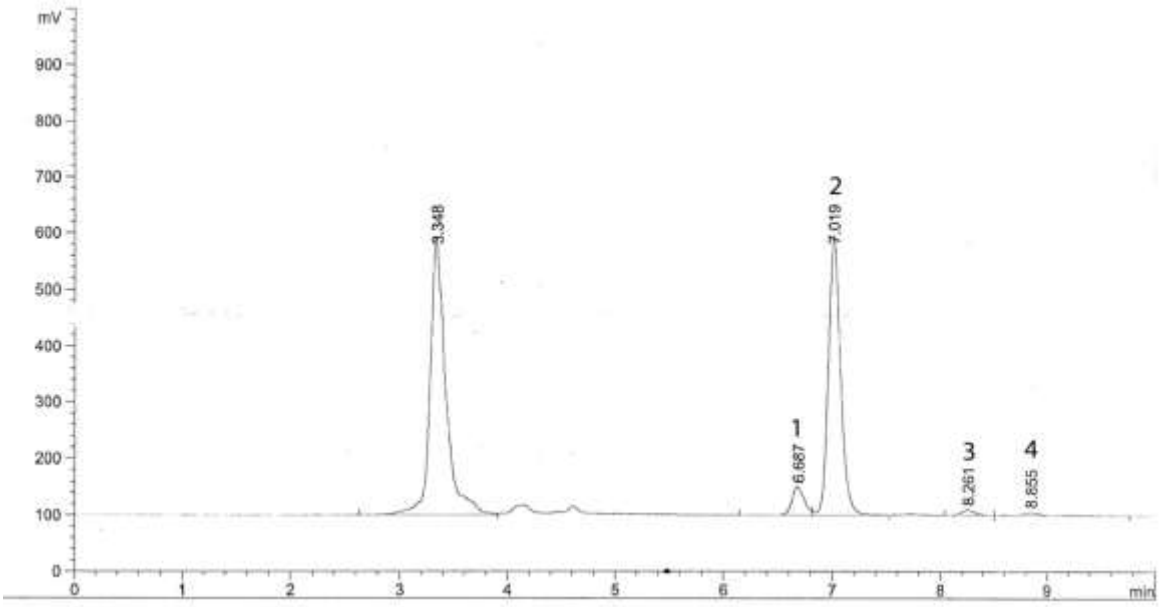

Fig. 2: Chromatogram of stevioside, rebaudioside A, rebaudioside C and dulcoside A in stevia extract obtained using socletation with ethanol solvent

Stevioside, rebaudioside A, rebaudioside C and dulcoside A concentration on stevia with socletation extraction as follows: 12.50%, 0.80%, 0.30% and 0.10%.

Analysis of stevioside, rebaudioside A, rebaudioside C and dulcoside A in stevia extract obtained by percolation method with ethanol solvent, obtained chromatogram as follows:

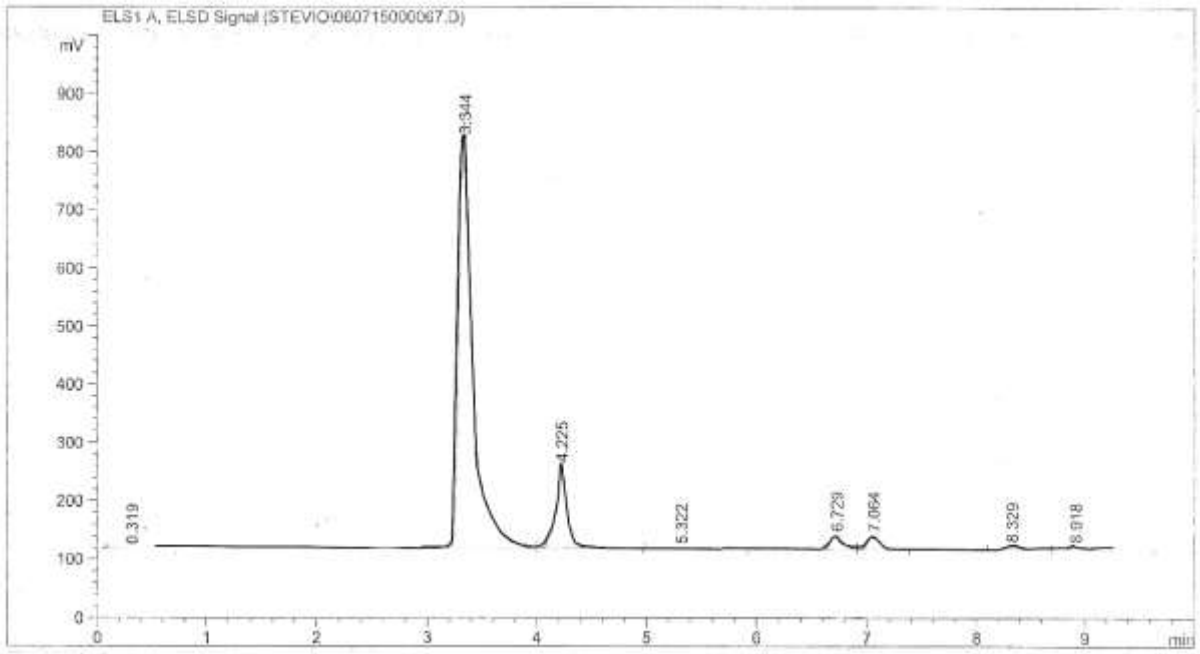

Fig. 3: Chromatogram of stevioside, rebaudioside A, rebaudioside C and dulcoside A in stevia extract obtained using percolation with ethanol solvent

Stevioside, rebaudioside A, rebaudioside C and dulcoside A concentration on stevia with percolation extraction with ethanol solvent as follows: 7.50%, 0.60%, 0.20% and 0.05%.

Analysis of stevioside, rebaudioside A, rebaudioside C and dulcoside A on stevia extract obtained using maceration and stirring at boiling temperature of ethanol, then the result was analyzed by using HPLC-ELSD and was obtained chromatogram and concentration of stevioside, rebaudioside A, rebaudioside C and dulcoside A as follows: 13, 50%, 0.90%, 0.50% and 0.20%.

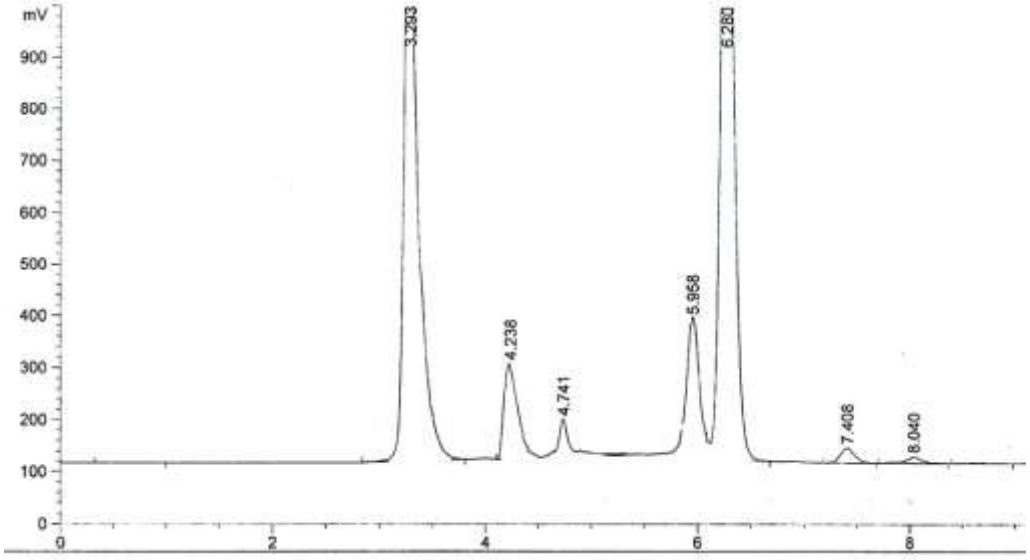

Fig. 4: Chromatogram of stevioside, rebaudioside A, rebaudioside C and dulcoside A in stevia extract obtained using maceration and stirring with ethanol solvent at its boiling temperature.

Analysis of stevioside, rebaudioside A, rebaudioside C and dulcoside A on stevia extract by percolation extraction method with methanol solvent, then extract was analyzed by HPLC-ELSD and we obtained chromatogram and concentration of stevioside, rebaudioside A, rebaudioside C and dulcoside A as follows: stevioside levels, rebaudioside A , rebaudioside C and dulcoside A as follows: 13.60%, 0.94%, 0.50% and 0.22%.

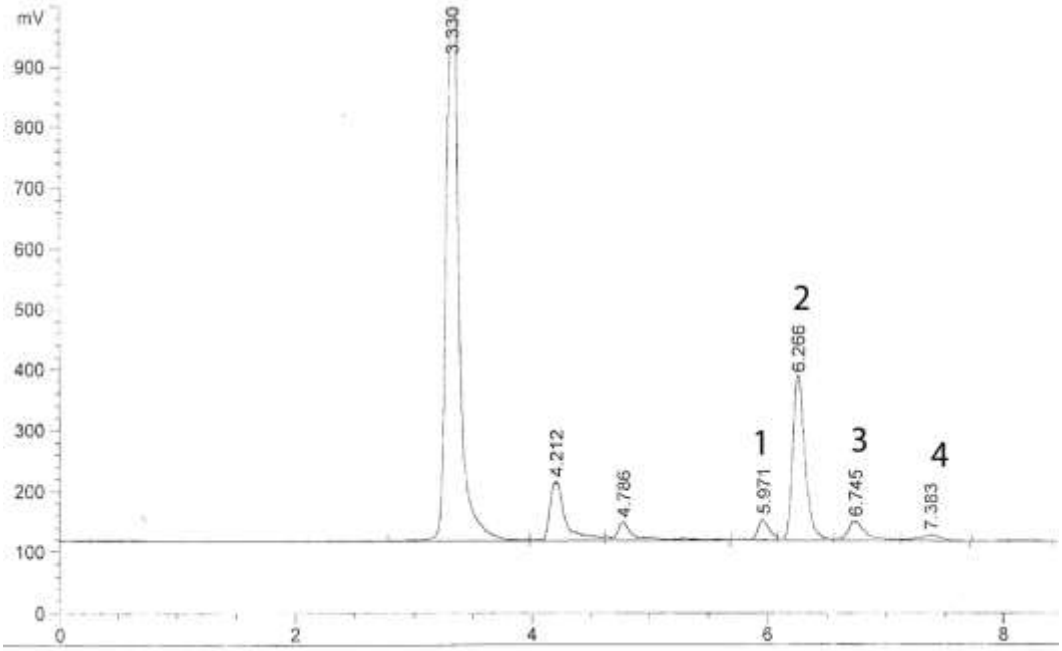

Fig. 5: Chromatogram of stevioside, rebaudioside A, rebaudioside C and glucoside A in stevia extract obtained using socletation with methanol solvent.

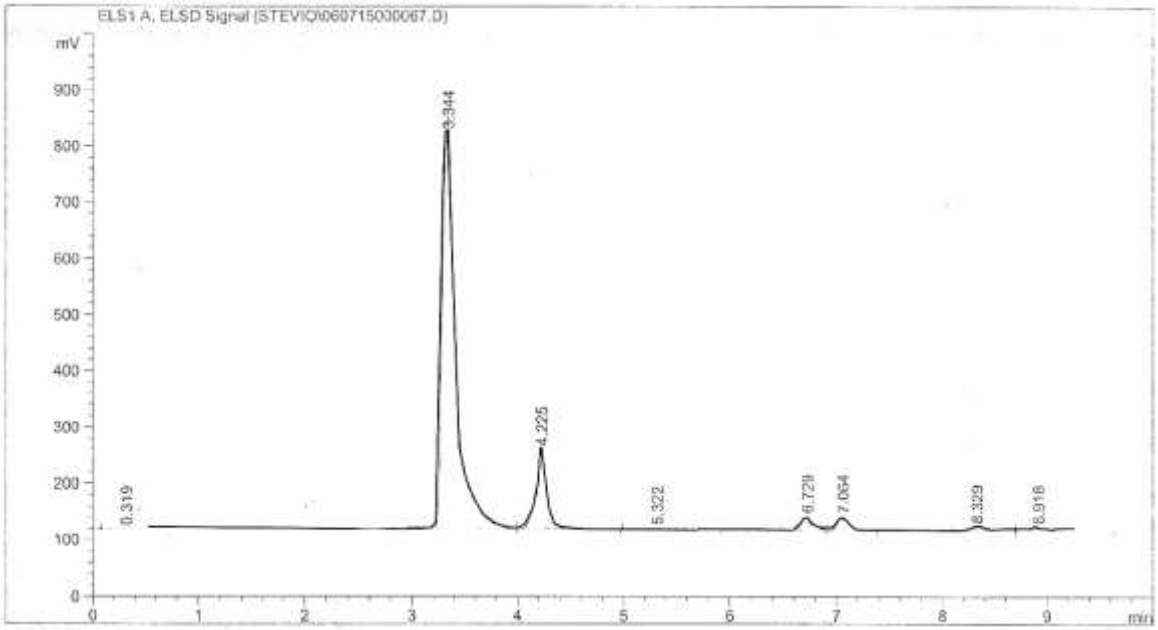

Fig. 6: Chromatogram Analysis of the content of stevioside, rebaudioside A, rebaudioside C and dulcoside A in stevia extract obtained using percolation with methanol solvent.

Concentration of stevioside, rebaudioside A, rebaudioside C and dulcoside A as follows: 8.60%, 0.64%, 0.44% and 0.20%.

Analysis of stevioside, rebaudioside A, rebaudioside C and dulcoside A in stevia extract obtained using maceration and stirring method at boiling temperature of methanol, then obtained extract was analyzed by HPLC-ELSD and we obtained chromatogram and concentration of chromatogram and levels of stevioside, rebaudioside A, rebaudioside C and dulcoside A as follows: 13, 91%, 1.00%, 0.56% and 0.30%.

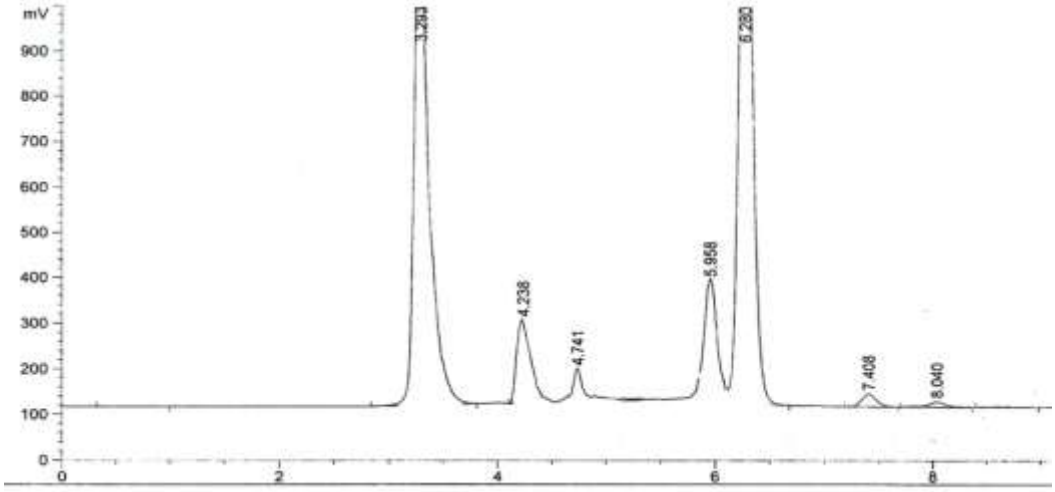

Fig. 7: Chromatogram of stevioside, rebaudioside A, rebaudioside C and dulcoside A in stevia stevia extract obtained using maceration method and stirring methanol solvent at its boiling temperature.

From three different extraction methods (socletation, percolation and maceration with stirring at boiling temperature) the highest level of steviol glycoside was found in the maceration extract. Also, extraction using methanol solvent exhibit highest level of steviol glycoside compared to the other solvent.

CONCLUSION

Based on HPLC-ELSD analysis the highest concentration of stevioside, A, rebaudioside C and dulcoside A was found in stevia extract using maceration with stirring at solvent boiling temperature using methanol. Maceration was done repeatedly with a new solvent and heat, making it easier to dissolve stevioside, rebaudioside A, rebaudioside C and dulcoside A compounds from stevia.

We suggest to obtain the highest level of steviol glycoside from stevia can be done by using repeated methanol maceration with stirring and at solvent boiling temperature. However, if stevia extract is to be used for consumption it is better to be done by maceration with ethanol solvent, not only because with the concentration difference is small, but also methanol residue is very toxic.

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