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# EFFECTS OF HARVESTING TIME, DRYING, EXTRACTION AND PURIFICATION METHODS ON SWEETENERS EXTRACTED FROM *Stevia rebaudiana* Bertoni

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## ABSTRACT

*Stevia rebaudiana* Bertoni plant was cultivated in pots at greenhouse and open field at farm near Alexandria, Egypt. Leaves of the plant were collected monthly during growth period (7 months) and dried with three different methods. Sweeteners in the leaves were first determined with IR. Proximate composition was also determined. Dried leaves were extracted and purified with different methods. Extraction and purification efficiency of each method were monitored with TLC and HPLC determination. The results obtained revealed that high level of carbohydrate obtained at 4 months of growth which seems to be the most proper harvesting time, drying in open air at 19-31°C for 24 hrs daily had adversely affected the carbohydrates content in leaves. Potassium was the highest content of mineral content in dry leaves. The highest concentration of stevia sweeteners in leaves obtained at 4 months age. The highest amount of stevioside and RA were obtained in MeoH extract. Purification of stevia extracts were achieved by using neutral alumina or active carbon which removed all coloured polar constituents. Finally purified stevia sweeteners component were separated and identified by TLC and HPLC.

## INTRODUCTION

*Stevia rebaudiana* Bertoni is a small perennial shrub of the Compositaé family whose leaves contain different diterpene glycosides. All those compounds are non caloric sweeteners (Shaffert and Chebotar, 1994 and Chalapalhi *et al*., 1997). The leaves of this plant contains stevioside (Stevia sweetener), diterpene glycoside, which is the major sweetener in the leaves, it has the property of being 300 times sweeter than sucrose (Das *et al.* 1992; Hanson and De Oliveira, 1993 and Richard, 1996). In addition to stevioside, other similar glycosides such as rebaudioside A, B, C, D and E and ducloside A (Kinghorn and Soejarto, 1985) are present in stevia leaves in small quantities. Harvest date had highly significant effects on plant height, leaf length, leaf width, leaf thickness, tiller number, fresh and dry weight of stems and leaves, dry leaf weight and content of sweet components (Shyu *et al.,* 1994). The leaves are harvested about 4 months after planting out. The leaves are dried in the shade for 3-4 days. Under favourable conditions the plants grow again and 3-4 cuts/year (Donalisio *et al.,* 1982). Metivier and Viana (1979), Chang and Huang (1981), Ahmed and Dobbersten (1982), Cernades and Pryluka (1985) and Striedner *et al.* (1991) studied that the extraction methods for obtaining sweeteners from leaves. They reported that dried leaves of *Stevia rebaudiana* are extracted with $CHCl_3$, ethanol, n-butanol, water, methanol or methanol : water. Adduci *et al.* (1987); Jakinovich

*et al.* (1990); Nishiyama *et al.* (1992) and Pistunov *et al.* (1993) studied the purification of stevia extracted.

In Egypt, there is a great interest on the strategical agriculture plan for new crops cultivation. It may reduce :Food Gap" especially in the area of sugar and sweeteners. Recently there is a great effort for natural sweeteners production. Therefore, the objectives of this work to study the chemical composition of stevia leaves during growth stage and also to investigate the effect of different factors such as harvesting time, drying, extraction, purification methods on sweeteners obtained.

## MATERIALS AND METHODS

*Stevia rebaudiana* Bertoni seeds were obtained from Tokiwa Shokubutsu Kagaku Laboratories Co. Ltd., Chiba, Japan. Seeds were cultivated in pots at green house [Sabahia Agricultural Research Center]. Cultivation of seeds was carried out in April. At the same time seeds of *Stevia rebaudiana* Bertoni were cultivated in open field at private farm near from Alexandria. Leaves samples were collected monthly during growth period (stevia months) for analysis. An additional sample of dried leaves was supplied by Sigma-Aldrich Corporation, USA. Stevia sweeteners was also obtained from Stevia International Company for Agra industrial Projects (SKAP, Japan).

**Dry methods:** The stevia leaves were subjected to three different ways of drying, first the leaves were dried in electric oven (E. Schulz & Co. Inh. Franz. Skorezewsh KG) at 50°C. In the second way, leaves were sun dried at sun shine period for 5-6 hours each day with temperature of 29-31°C till reached the constant and required moisture content (9-11%). For the third way leaves were sun dried for all the day (24 hrs.) with temperature ranged from 19-31°C and relative humidity was ranged from 78.89-81.21% till the moisture content reached to 9-11%.

**Stevioside standard preparation:** Stevioside standard preparation was carried out according to Nishiyama *et al.* (1992) with some modification presented in Fig. 1. Moisture, protein, crude fat, ash and crude fiber contents (%) were determined according to AOAC (1990). The carbohydrate (%) was calculated by difference. The minerals content (Fe, Mg, K, Zn and Ca) was determined using Perkin-Elmer Atomic Absorption Spectrophotometer (Model 2380 A.A.S., England), Na was determined using the flame Analyzer Photometer (Gallenkanp. FGA 330, England) according to A.O.A.C. (1990).

**Infrared spectra of sweeteners in leaves**: Dried *Stevia rebaudiana* Bertoni leaves in different growth stages were ground on a 1 mm screen, prepared as pellets with KBr and analyzed by IR spectrophotometer (Perkin Elmer 1420). The absorption bands (Vmax) are given in wave numbers ($cm^{-1}$) [Nishiyama *et al.* 1992].

Fig 1

Extraction of stevia leaves were carried out with methanol (Yamazaki *et al.,* 1991), with MeoH : $H_2O$ (4 : 1) as Kinghorn *et al.* 1984 and with water (Nishiyama *et al.,* 1992).

Samples (20 µl) of stevia extract were applied on TLC plate of silica gel GF 245 (250 µm x 20 x 10 cm). The plates were developed with three different solvent systems according to Yamazaki *et al.* (1991); the plates were dried and visualized, according to Fujinuma *et al.* (1986). Identification of bands was carried out according to the Rf values obtained by Yamazaki *et al.* (1991).

**Identification and quantitative analysis of stevia sweeteners by HPLC in stevia leaves extract:** Stevia leaves extract was separated and identified on HPLC as the following: Stevioside and other sweet component standard as prepared above were filtered through a millipore membrane (13 mm diameter, 0.5 µm pore size) were injected straight a way for chromatography with stevioside standard as internal standard. Different extracts of stevia leaves were injected for chromatography Acetonitrile used in this study as mobile phase was HPLC grade (Fisons Co. England). HPLC separation was carried out on Shimadzu (SPD-6 AV) with UV-Vis spectrophotometer, detector of LC-GA and an Alex C-R 4A recorder. The operating conditions for HPLC were as following: Column, Zorbax $NH_2$, 25 cm x 0.4 mm I.D. (Dupont, Wilmington, DE, U.S.A.), eluting solvent acetonitrile : water (84 : 70 v/v), pH 5 adjusted with $H_3PO_4$, flow rate 2 ml/min, wave length of UV detector, 210 nm, recorder chart speed 20 mm/min at ambient temperature (25°C). All this conditions are completely as the same conditions used by Makapugay *et al.*, (1984). For each sample identification and quantification the retention time obtained by Makapugay *et al.* (1984), and area under each peak used for calculation the percent of each compound.

**Purification of stevia sweeteners.** Silica gel column (22 mm Id.) was prepared as follow: slurry of silica gel (AD, 100-250 mesh) 2.5 g in methanol, added to column, when gel settled, its surface was covered with disc of filter paper. The column was washed with 10 ml MeoH, then rinsed with 50 ml, 20% MeoH, followed by 80% MeoH. The crude extract of stevia leaves was passed through silica gel column at flow rate of 5 ml/min. The collected eluent was evaporated to dryness at 40°C in rotary evaporator under reduced pressure. (Yamazaki *et al*., 1991).

**Alumina oxide column:** The crude extract was passed through pasteur pipette packed with 2 g of activated neutral aluminum oxide (BDH chemicals Ltd. Poole England) and eluted with 20 ml MeoH : $H_2O$ (1 : 1). Samples were prepared for preliminary TLC by removing solvent under reduced pressure and analyzed by HPLC. [Kinghorn *et al.* 1984].

**The active carbon column:** A column (22 mm) was packed with active carbon powder (The British Drug Houses LTD. B.D.U. Laboratory, Chemical Division, Poole, England). The aqueous extract were passed through active carbon column, then the eluent was evaporated to dryness and kept for TLC and HPLC analysis. Carbon column washed with 25 ml ethanol 50% and then

with 25 ml ethanol 80%, eluents obtained by ethanol 50% and 80% was evaporated and analyzed by TLC and HPLC. [Chang and Huany (1981)].

## RESULTS AND DISCUSSION

*Stevia rebaudiana* Bertoni was cultivated in green house and open field in order to investigate proper time of harvesting, as well as the seasonal variations of its monthly proximate composition during plantation period. Table (1) showed the changes occurred in proximate composition at different period of growth stages. It is clear that high level of carbohydrate can be obtained at 4 months of growth which seems to be the most proper harvesting time as plant began blooming. This result is in agreement with that reported by Chen *et al.* (1979), who concluded that when plant began blooming, the major sweetener compound, stevioside content in leaves reached its maximum (8.7%) and consequently the maximum yield of leaves can be obtained (3.395 kg/ha) which seems to be the most beneficial time for harvesting.

**Table (1): Changes of proximate composition in *Stevia rebaudiana* Bertoni leaves during different period of growth.**

| Growth period month | Proximate composition % | | | | | |
|---|---|---|---|---|---|---|
| | Moisture | Protein* | Crude fat* | Ash* | Crude fiber* | Carbo-hydrate* |
| 2 | 82.97 | 13.98 | 5.26 | 17.83 | 10.24 | 52.69 |
| 3 | 80.04 | 12.87 | 5.01 | 14.59 | 10.86 | 56.67 |
| 4 | 79.71 | 12.46 | 4.98 | 13.45 | 10.94 | 58.17 |
| 5 | 66.58 | 14.66 | 4.21 | 15.08 | 12.38 | 53.67 |
| 6 | 49.67 | 14.92 | 3.85 | 17.21 | 13.06 | 50.36 |
| 7 | 14.55 | 15.04 | 3.80 | 18.73 | 14.88 | 46.55 |

*** Calculated on dry weight basis and carbohydrates by difference.**

**Effect of drying method on sweeteners in stevia leaves**: The effect of different drying method on proximate composition of leaves harvested after four months of growth are shown in Table (2). Dry in open air at 19-31°C for 24 hrs. daily had affected the carbohydrate content which decreased 4% comparing with fresh leaves. Drying by electric oven at 50°C or at sun shine at 29-31°C had slightly decreased the carbohydrate content (0.25-0.73%). These results are in agreement with the results obtained by Cheng *et al.* (1981) who obtained the best results when they harvested the stevia plant on sunny day and it was dried at sun shine time.

**Minerals content of stevia leaves from different sources:** Potassium was the highest content of mineral and reached its maximum content in leaves produced in green house (3.537%) followed by open field stevia leaves (3.042%) and amounted to three folds of the potassium content of imported leaves (1.202%) (Table, 3). On the other hand the leaves contained small amounts of iron, sodium and zinc comparing with potassium and calcium.

**Table (2): Effect of drying method on a proximate composition of *Stevia rebaudiana* Bert. Leaves (4 months old).**

| Drying methods | Proximate composition (%) | | | | | |
|---|---|---|---|---|---|---|
| | Moisture | Proteir | Crude fat* | Ash* | Crude fiber* | Carbohydrate* |
| None (Fresh leaves) (19-31°C) | 79.71 | 12.46 | 4.98 | 13.45 | 10.94 | 58.17 |
| Sun at 19-31°C (24 hrs./day) | 10.72 | 12.40 | 4.79 | 15.61 | 12.80 | 54.40 |
| Sun at 29-31°C (5 hrs./day) | 10.91 | 12.10 | 4.61 | 13.74 | 11.64 | 57.91 |
| Oven at 50°C | 9.09 | 12.29 | 4.28 | 14.02 | 11.99 | 57.42 |

*** Calculated on dry weight basis.**

**Table (3): Minerals content of *Stevia rebaudiana* Bertoni leaves samples from different sources (% dry weight basis).**

| Stevia leaves samples | Iron | Calcium | Potassium | Sodium | Magne-sium | Zinc |
|---|---|---|---|---|---|---|
| *Green house | 0.068 | 1.606 | 3.537 | 0.176 | 2.512 | 0.004 |
| *Open field | 0.182 | 2.091 | 3.042 | 0.152 | 1.990 | 0.003 |
| Imported | 0.173 | 2.180 | 1.202 | 0.063 | 1.432 | 0.003 |

*** Stevia leaves at 4 months of growth period.**

**Infrared spectrometry:** Infrared spectrometry was used to study the presence and concentration of the sweeteners in the *Stevia* leaves at different periods of growth with reference to the crude standard stevioside as indicated in Figure (2) The highest concentration of stevia sweeteners appeared in the plant sample of 4 months age (Figure 3) which indicated that this age is the best period for harvesting the leaves to obtain the highest yield of the sweeteners. Nishiyama *et al.* (1992) stated that Near infrared reflectance spectrophotometry (NIRS) system has been shown to be a very useful technique for measuring the percentage of sweeteners in powdered stevia leaves, due to the reduced analysis time required, the small amount of ground material needed and the less cost.

**Effect of different solvents on the extraction of stevioside and rebaudioside A as the major (stevia sweeteners) in stevia leaves:**

Dry leaves of stevia from different sources were extracted with three different solvents namely; MeoH, MeoH: $H_2O$ (4:1 v/v), and $H_2O$. Stevioside and rebaudioside A (R.A), as the major components of stevia sweeteners, were determined by HPLC analysis in each extract. MeoH, MeoH : $H_2O$ and $H_2O$ extracts.

Fig 2

fig 3

The results are shown in Table (4). It is clear that the highest amount of stevioside and R.A were obtained in MeoH, extract followed by MeoH : $H_2O$ extract and $H_2O$ extract which gave the lowest amount of stevioside and R.A. At the same time, the highest level of stevioside and R.A were obtained in stevia leaves cultivated in green house (sun dried) with the three solvents used in the extraction comparing with other stevia leaves from other sources. Hashimoto *et al.* (1978) studied the stevioside and rebaudioside A content in dry stevia leaves extract. They concluded that these contents ranged from 2.0 to 7.7% and 0.8 to 2.9% for stevioside and rebaudioside A, respectively. On the other side Morita (1986) reported thatmethanol is preferable to extract the rebaudioside A together with stevioside. Also, the results obtained by HPLC analysis for each stevia sweeteners extracted from dry leaves at 2, 3, 4, 5, 6 and 7$^{th}$ months of growth stage during cultivation in green house.

**Table (4): Effect of three solvents on extraction of stevioside and rebaudioside A (R.A) from dry stevia leaves of different sources as determined by HPLC.**

| Stevia leaves source | MeoH | | MeoH : $H_2O$ | | $H_2O$ | |
|---|---|---|---|---|---|---|
| | ST* | R.A** | ST | R.A | ST | R.A |
| ***Green house (sun dried at 29.31°C, 5 hrs./day) | 22.11 | 12.20 | 17.99 | 9.83 | 16.98 | 8.40 |
| ***Green house (oven dried at 50°C) | 20.03 | 11.78 | 16.88 | 9.47 | 15.23 | 8.74 |
| Open field (oven dried at 50°C) | 17.85 | 9.04 | 14.09 | 8.21 | 13.20 | 7.73 |
| Imported | 19.01 | 8.30 | 16.77 | 7.44 | 15.29 | 7.01 |

*** ST = Stevioside (%) ** R.A = Rebaudioside (%). *** Stevia leaves at 4 months old.**

Figure (4) showed that the same trend of results obtained in Table (5) where MeoH extraction gave the highest yield of stevia sweetener. In the same time, stevia sweeteners content increased after 3 months (27.50, 23.51, and 15.25 by MeoH, MeoH: $H_2O$ and $H_2O$ extraction respectively) and reached to their maximum after 4 months of growth (38.96, 31.39 and 25.96% by MeoH, MeoH : $H_2O$ and $H_2O$ extraction respectively. After completely blooming at 5 months of growth the stevia sweeteners content decreased to 34.93, 49.12 and 45.49% of the sweetener percent obtained at 4 months of growth as extracted by MeoH, MeoH : $H_2O$ and $H_2O$ extraction respectively). From these results it can be concluded that the appropriate time for stevia plant harvesting is after 4 months of growth stage. In the same time, Table (6) shows the effect of harvesting time on different stevia sweeteners compounds content as extracted with MeoH and determined with HPLC analysis.

**Fig4**

**Table (5): Effect of different solvent extraction on stevia sweeteners from dry stevia leaves green house at different growth period as determined by HPLC.**

| Growth stage (month) / Solvent type | Stevia sweeteners (%) | | | | | |
|---|---|---|---|---|---|---|
| | 2 | 3 | 4 | 5 | 6 | 7 |
| MeoH | 17.91 | 27.50 | 38.96 | 25.12 | 16.07 | 11.79 |
| MeoH : $H_2O$ | 15.53 | 23.51 | 31.39 | 15.97 | 10.80 | 8.20 |
| $H_2O$ | 11.04 | 15.25 | 25.96 | 14.15 | 6.57 | 5.17 |

**Table (6): Effect of harvesting time on different sweeteners percent of dry* stevia leaves as extracted with MeoH and determined by HPLC.**

| Sweeteners | Harvesting time (months) | | | | | |
|---|---|---|---|---|---|---|
| | 2 | 3 | 4 | 5 | 6 | 7 |
| Ducloside A % | 0.08 | 0.08 | 0.06 | 0.06 | 0.06 | 0.08 |
| Stevioside % | 9.15 | 15.28 | 20.03 | 12.34 | 6.89 | 5.48 |
| Rebaudioside A % | 5.28 | 7.32 | 11.78 | 8.66 | 5.92 | 4.06 |
| Rebaudioside C % | 3.36 | 4.80 | 6.63 | 4.06 | 3.19 | 2.15 |
| Rebaudioside E % | 0.02 | ND** | 0.46 | ND | 0.01 | 0.01 |
| Total sweeteners % | 17.90 | 27.48 | 38.96 | 25.12 | 16.07 | 11.79 |

*** Stevia dry leaves obtained from green house and dried up to (9.1-10.2%) moisture before extraction.**
**** ND = not detected.**

It is clear that dulcoside A, stevioside, R.A, R.C and R.E. content was increased after three months and reached the highest amount after four months (Fig. 5). In the same time, Fig. (6) illustrated that the highest amount of sweeteners in leaves and stem were obtained after 4 months of growth and that is the most suitable time for harvesting. Also, it can be concluded that the major sweetening compounds present in stevia sweeteners, extracted from 4 months old plants, were stevioside and R.A. which represented 51.41 and 30.14% respectively of total sweeteners percent. Pryluke and Cerhades (1985) found that the better solvent used for extraction of stevioside was methanol and monodimensional chromatography using chloroform: methanol : $H_2O$ (90 : 95 : 9 v/v). Striedner *et al.* (1991) used cold methanol for extraction of stevia sweeteners from *Stevia rebaudiana* Bert. while Nishiyama *et al.* (1992) used water to extraction stevia sweeteners.

**Purification of stevia sweeteners extracted from stevia leaves:**

Some of the accompanying substances in stevia sweeteners extract can be precipitated from the extracts by means of oxides or hydroxides of calcium, magnesium or aluminum. Further purification can be achieved through precipitation by means of organic solvents such as ether or organochlorine compounds, but normally the sweeteners is attached to adsorbents such as polymeric resins or activated carbon and then desorbed from these again by means of hydrophilic solvents. This may be followed by crystallization (Higginbotham, 1983). In this study many trials have been done for the purification of stevia sweeteners extracts. Some of these trials did not achieve the goal for obtaining pure stevia sweeteners free of pigments or coloured polar constituents, gummy substances and the recovery of the pure stevia sweeteners will be at least 90%. The first trial of purification was carried out by using some of organic solvents such as ethyl acetate. In the same time, silica gel column as a method for purifying the sweeteners extract was tried. The first successful trial was achieved by using neutral alumina oxide which removed all coloured polar constituents. The sweeteners were eluted and identified on TLC and was determined by HPLC. By this procedure, pigments were removed very effectively and recoveries of the two major stevia sweeteners; stevioside and rebaudioside A were found to be 95 and 96%, respectively of its primary content in extract. These results are in agreement with these obtained by Kinghorn *et al.* (1984) who showed that the passage of stevia sweeteners extract in n-BuOH soluble fractions through alumina column enabled the removal of many colored polar constituents while allowing the elution of the ent-kaurene glycoside.

Also, a simple method for purifying sweeteners extract of dry stevia leaves was developed in which active carbon was used. The adsorbed sweeteners on active carbon were then desorbed from active carbon with an aqueous ethanol. The elute colourless and pure was obtained. Chang and Huang (1981) reported that purification on active carbon column gave an elute of pure and high stevioside concentrate. Also, Nishayama *et al.* (1992) used active carbon to purify stevia sweeteners. Purified stevia sweeteners were dried under vacuum with rotary evaporator at 50-55°C. The dried sweeteners were dissolved in hot methanol and kept overnight at –5°C to crystallize. Then separated with filtration and kept dry in desiccator over calcium chloride.

Fig 5

**Fig6**

**Identification of stevia sweeteners by TLC:**

Pure stevia sweeteners obtained were separated and identified by TLC according to Yamuzaki *et al.* (1991). The better separation for stevia sweetener compounds was obtained by using solvent system ethyl acetate : acetic acid : water (8 : 3 : 2) as it indicated in Figure (7). It is clear that five diterpene glycoside namely steviolbioside, stevioside, rebaudioside A (RA), R.E and R.C were identified in stevia sweeteners. Fulias *et al.* (1989 and Striedner *et al.* 1991 separated the stevia sweeteners on TLC and obtained the same results. Pure stevia sweeteners were also separated and identified by HPLC as indicated in Figure (5).

**Fig 7**

## REFERENCES

Adduci, J.; D. Buddhasukh and B. Ternai (1987). Improved isolation and purification of stevioside. J. Sci. Soc. Thailand, 13: 179-183.

Ahmed, M.S. and R.H. Dobberstein (1982). *Stevia rebaudiana*. III. High performance liquid chromatographic separation and quantitation of rebaudiosides B, D and E, dulcoside A, and steviolbioside. Journal of chromatography, 245: 373-376.

A.O.A.C. (1990). Official Methods of Analysis of the Association of Official Analytical Chemists, Kenneth Helrich (Ed.) 15th Ed. Association of Official Analytical Chemists Arlington, Virginia, 22201 U.S.A.

Cernadas, R.R. and M. Pryluka (1985). A method for the obtention of stevioside from leaves of *Stevia rebaudiana* Bert. Revista-de Agroquimica-Y. Technologia de Alimentos, 25: 268-272.

Chalapalhi, M.V.; S. Thimmegowda; S. Sridhara; VR. Ramakrishna-Parame. and TG. Prasad (1997). Natural non-caloric sweetener stevia (*Stevia rebaudiana* Bertoni) a future crop of India. Crop Research, 14: 247-350.

Chang, W.H. and S.A. Huang (1981). A simple method for steviosides purification with active carbon. Recent Advances in Food Science and Technology, 22:484-496.

Chen, K.; S.L. Tang; T.R. Chang and W.H. Chang (1979). Studies on the cultivation of stevia and seasonal variation of stevioside content. Food Sci., 6: 8-13, (Taiwan)

Cheng, T.F.; W.H. Chang and T.R. Chang (1981). A study on the post-harvest changes in steviosides contents of stevia leaves and stems. Natl. Sci. Counc. Monthly, ROC, 9: 775-782.

Das, S.; A.K. Das; R.A. Murphy; I.C. Punwani;M.P. Nasutionand A.D. Kinghorn (1992). Evaluation of the cariogenic potential of the intense natural sweeteners stevioside and rebaudioside A. Caries-Res., 26: 263-266.

Donalisio, M.G.R.; F.R. Duarte; A-D.A. Pinto and C.I. Souza (1982). *Stevia rebaudiana*. Agronomico., 34: 65-68.

Fujinuma, K.; K. Saito; M. Nakazato; Y. Kikuchi and T. Nishima (1986). Thin layer chromatographic detection and liquid chromatographic determination of stevioside and rebaudioside A in beverages and foods following reverse phase column chromatography. J. ASSOC. oFF. Anal. Chem., 69:799-802

Fulias, F.; J. Kim; C.M. Compadre and A.D. Kinghorn (1989). Separation of natural product sweetening agents using overpressured layer chromatography. J. of Chromatography, 464: 213-219.

Hanson, J.R. and B.H. De Oliveira (1993). Stevioside and related sweet diterpenoid glycosides. Natural Product Reports, 10: 301-309.

Hashimoto, Y.; M. Moriyasu; S. Nakamura; S. Ishiguro and M.M. Komero (1978). High performance liquid chromatographic determination of stevia components on a hydrophilic packed column. Journal of chromatography, 161: 403-405.

Higginbotham, J.D. (1983). Recent Developments in Non-nutritive Sweeteners. In: Developments in sweeteners" Ed. Grenby, T.H.; Parker, N.J. and Lindley, M.G. 2. Applied Science Publishers, London, New York.

Jakinovich, W.; C. Moon; Y.H. Choi and A.D. Kinghorn (1990). Evaluation of plant extracts for sweetness using the Mongolian gerbil. J. Nat. Prod. 53: 190-5.

Kinghorn, A.D.; D.D. Soejarto; N.P. Nanayakkara; C.M. Compadre, H.C. Makapugay; J.M. Hovanec-Brown; P.J. Medon and S.K. Kamath (1984). A phytochemical screening procedure for sweet ent-kaurene glycosides in the genus stevia. J. Nat. Prod., 47: 439-44.

Kinghorn, A.D. and D.D. Soejarto (1985). Current Status of Stevioside as a Sweetening Agent for Human Use. In Economic and Medicinal Plant Research. Vol. I. Ed. by H. Wagner, H. Hikino and N.R. Farnsworth. Academic Press. London.

Makapugay, H.C.; N.P.D. Nanayakkara and A.D. Kinghorn (1984). Improved high-performance liquid chromatographic separation of the *Stevia rebaudiana* sweet diterpene glycosides using linear gradient elution. J. of chromatography, 283: 390-395.

Metivier, J. and A.M. Viana (1979). Determination of microgram quantities of stevioside [sweetening compound] from leaves of *Stevia rebaudiana* Bertoni by two dimensional thin layer chromatography. J. of Experimental Botany, 30: 805-810.

Morita, K. (1986). Preparation of stevioside sweetener composition. Japanese Patent, 61: 202 - 667

Nishiyama, P.; M. Alvarey and L. Vieira (1992). Quantitative analysis of stevioside in the leaves of *Sevia rebaudiana* by Near Infrared reflectance spectroscopy. J. Sci. Food Agric., 59: 277-281.

Piskunov, N.V.; V.A. Polyakov; A.V. Oreshchenko; N.F. Beresten; V.F. Zubento; N.N. L'enko; B.D. Chudnovskili and T.K. Yavorskaya (1993). Manufacture of a sweetener from *Stevia rebaudiana* Bertoni leaves. USSR. Patent C.F. FSTA. 25, IT57 (1995).

Pryluka, M. and R.R. Cernades (1985). Determination of the stevioside content in leaves *Stevia rebaudiana* Bertoni. Revislade-Agroguimica-Y-Tech- nologic-de Alimentos 25, 611-614 C.F. HORTCD 3305172 (1986).

Richard, D. (1996). The secret of stevia. The Natural sweetener. Health world on line. C.F. File : C :\ My document\My pictures\secret. htm.

Shaffert, E.E. and A.A. Chebotar (1994). Structure, topography and ontogeny of *Stevia rebaudian* (Asteraceae). Botanicheskii-Zhurnal, 79: 38-48. C.F. HORTED 0300487 (1996).

Shyu, Y.T.; S.Y. Liu; H.Y. Lu; W.K. Wu and C.G. Su (1994). Effects of harvesting dates on the characteristics, yield and sweet components of stevia (*Stevia rebaudian* Bertoni) lines. Journal of Agricultural Research of china, 43:29-39.

Striedner, J.; E. Gutjahr; F.C. Czygan and G. Braunegg (1991). Contribution to the biotechnological production of sweeteners from *Stevia rebaudian* Bertoni. I. A method for the serial analysis of diterprene

glycosides by HPLC. II. Induction of the nutrient medium and the analysis of small amounts of Small amounts of stevioside stevioside Acta-Biotechnol, 11: 495-504.

Yamazaki, T.; H.E. Flores; K. Shimomura and K. Yoshihira (1991). Examination of steviol glucosides production by hairy root and shoot cultures of *Stevia rebaudiana.* Journal of Natural Products, 54: 986-992.

## تأثير ميعاد الحصاد وطرق التجفيف والاستخلاص والتنقية على المحليات من نبات الاستيفيا

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زرع نبات الاستيفيا داخل الصوبة في أصص وأيضا في الحقل في منطقة قريبة من الإسكندرية . تم تجميع أوراق النبات كل شهر خلال فترة النمو وتم دراسة التجفيف بثلاث طرق . وتم تقدير المحليات مباشرة في الأوراق بالـ I.R وأيضا تم تقدير التركيب الكيماوي ثم استخلاص المحليات من الأوراق الجافة بطرق مختلفة وأيضا تمت تنقية المستخلص وتقدير كفاءة طرق الاستخلاص والتنقية بواسطة الـ TLC والـ HPLC . وأوضحت النتائج أن المستويات العالية من الكربوهيدرات تم الحصول عليها بعد أربعة أشهر من النمو والتي تعتبر أنسب وقت للحصاد . وقد أثر التجفيف في الهواء المفتوح على درجة حرارة ١٩-٣١°م تأثيراً سلبياً على محتوى الكربوهيدرات في الأوراق . وأن البوتاسيوم يمثل أعلى محتوى من المعادن في الأوراق الجافة . وأعلى تركيز من المحليات في الأوراق تم الحصول عليه بعد أربعة أشهر من النمو . كذلك أعلى كمية من الاستيفيوزيد و R.A وهما يمثلان أهم المحليات الموجودة في الاستيفيا تم الحصول عليها بواسطة الاستخلاص بالميثانول . أيضا تمت إزالة المواد الملونة القطبية بكفاءة بواسطة أكسيد الألومنيا المتعادلة أو الفحم النشط . وتم أيضا فصل مركبات المحليات المختلفة والتعرف عليها وتقديرها كمياً بواسطة كل من TLC والـ HPLC .