Ultrasound Extraction of Active Principles with Hypoglycaemic Activity from Medicinal Plants

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Intensification is a secure and worthy method of improving either a rather lengthy (time consuming) or an energy intensive (far from normal conditions) process, searching for the increase of at least one of the major parameters governing it: the kinetic, through the partial transfer rates, the interfacial area or the driving force, seen as the distance from the actual state of the process and its equilibrium. Ultrasound assisted extraction acts primarily upon the kinetic of the extraction process, and secondarily upon the interfacial area, through the eventual disintegration of larger particles. Compared with maceration, infusion or decoction, it improves the process decreasing both operating time and temperature while increasing the extraction yield. This intensive technique, biochemically safe unless overexposure, was used to obtain active principles with hypoglycaemic activity (sweet diterpenic steviol glycosides) from a medicinal plant with high economical potential: *Stevia rebaudiana* leaves. Distilled water or a mixture of water and ethanol in several ratios were employed as solvent. The active principles were quantified by HPLC. An Artificial Neural Network was used to model the ultrasound assisted extraction, due to its ability to cope with complex processes and superior generalization capacity.

Keywords: Ultrasound assisted extraction, HPLC, Medicinal plants, Stevioside, *Stevia rebaudiana Bert.*, Artificial Neural Network.

1. Introduction

Stevia rebaudiana, native from Paraguay, is used as herbal sweetener for over 1500 years. Extracts of *Stevia rebaudiana* are part in weight-loss programs because of its ability to reduce the cravings for sweet and fatty foods, to treat the diseases diabetes, hypoglycaemia, candidasis, high blood pressure, skin abrasions and inhibiting growth and reproduction of bacteria-like plaque (Gregersen *et al.*, 2004). *Stevia*'s greatest appears to be a natural alternative to artificial sweeteners (such as aspartame or sodium saccharin). The sweetness in Stevia rebaudiana is mainly attributed to two glycoside compounds: stevioside (3-10% of dry leaf weight) and rebaudioside A (1-3%) which can be up to 250 times sweeter than sucrose (Duke, 2006). The glycosides of *Stevia rebaudiana* leaves have been extracted using classical techniques: maceration, infusion or decoction, either requiring long processing time and low efficiency (maceration), or the facing thermal degradation (infusion and decoction) (Vinatoru, 2001).

In order to increase the productivity, several intensification techniques like ultrasonic waves, supercritical fluids or microwaves were associated with extraction of plant's compounds to improve the yield and quality of extracted products. From these, ultrasound assisted extraction seems to be economically most promising (inexpensive, simple and efficient), being employed to extract active compounds such saponins, steroids and triterpenoids from *Chresta spp.* about three times faster than with the traditional extraction methods (Schinor *et al.*, 2004). The main benefits of use of ultrasound in solid-liquid extraction include the increase of the extraction yield and faster kinetics (Kiel, 2007).

The purpose of the present experiment is to study the some parameters of extraction: dry leaves particle size, solvents nature, sample weight to solvent ratio (w/v), temperature, stirring and output power of the ultrasound and to determine the optimum domain in a reliable ultrasound assisted extraction protocol. High-performance liquid chromatography (HPLC) is used to separate and quantify the stevioside due to its high reproducibility, good linear range, ease of automation and ability to analyze the number of constituents in botanicals and herbal samples (Dacome *et al.*, 2005).

2. Materials and Methods

2.1 Reagents and plant material

Stevioside (S3572, assay ≥98% (HPLC), form: solid , colour: white, free soluble in water and ethanol, solubility H2O: >20 mg/mL, storage temp: 2-8°C, chemical name: 4α-13-[(2-O-β-D-Glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid β-D-glucopyranosyl ester, chemical formula: C38H60O18 – see Figure 1, molecular weight: 804.87) used as the standard chemical was obtained from Sigma Chemicals.

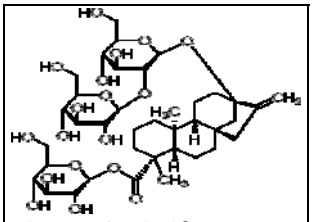

Figure 1 Stevioside – Structural formula

Chromatographic grade – double distilled water, HPLC grade acetonitrile, aqueous ethanol and distilled water was used as extraction solvents and mobile phase.

Dry leaves of *Stevia rebaudiana* were purchased from the Paraguay Medicine Market. They were stored in dark bags to protect them from humidity and light and, before each bunch of experiments, they were cut into pieces of the appropriate equivalent diameter.

2.1 Experimental setup

Two ultrasonic bath (Model T420 Elma and Model T460 Elma), both working at 35 kHz frequency and an output power of 70 W and 170 W respectively were employed. For stirring and heating a TK-22 Magnetic stirrer with heating was used. For chromatographic measurements, a HPLC system Agilent Technologies 1200 series model with UV-VIS detector was used. After the extraction process for all the methods, the filtrate was separated from the residual plant material by vacuum pump filtration.

2.2 Classic extraction

Maceration was used as reference for comparisons with the ultrasound assisted and hot extraction methods. Maceration was performed with 10 g of *Stevia rebaudiana* dry leaves with three particle sizes (0.315 mm, 2 mm and 6.3 mm) and different solvents:

distilled water and water/ethanol mixtures (55% and 70%) with different sample weight to solvent volume ratio (w/v): 1/10, 1/8, 1/5. The mixture was left at room temperature for 24 h in closed Erlenmeyer flasks, small volumes for analysis being probed at 5, 20, 45, 120, 240 minutes and at the end of the process. The hot extraction (infusion and decoction) was performed with 10 g of *Stevia rebaudiana* dry leaves having 0.315 mm equivalent diameter, at a sample weight to water volume ratio of 1/5 (w/v) and temperatures of 40 ^{0}C and 90 ^{0}C into Erlenmeyer flasks. For infusion (40 ^{0}C), samples were taken at 5, 10, 20, 25 and 35 minutes while for decoction (90 ^{0}C), samples were taken at 1, 2, 3, 5 and 8 minutes. Both maceration and hot extraction were performed with and without stirring.

2.3 Ultrasound assisted extraction

Samples of 10 g of dry leaves from *Stevia rebaudiana* with four particles sizes (0.315 mm, 2 mm, 6.3 mm and crushed dry leaves) were mixed with different solvents: distilled water and water/ethanol mixtures (55% and 70%) with different sample weight to solvent volume ratios: 1/10, 1/8, 1/5 (w/v) then placed into the ultrasound assisted extractors at room temperature. The ultrasonic baths were filled with liquid water into which glasses with samples were placed and sonicated for 1, 3, 5, 10, 15, 20, 25, 35, 40, 50 and 60 minutes at 70 W and 170 W respectively output power.

2.4 Dry residue analysis. Extractive values

According to the Romanian Pharmacopoeia (10th edition) approximately 2 g (2 ml) of extract was placed into a flat-bottomed glass dish (36 mm diameter and 28 mm height) covered to prevent evaporation of solvent before weighting. After weighting, the extract was dried in oven at 103 ^{0}C for 3 h. The content of extractive substances in the plant material was calculated from the mass of dry extract and the initial mass of plant subject to experiment. The concentration of extractive substances in the liquid extract was calculated from the mass of dry extract and the volume of liquid extract. The extractive value of the soluble compounds from the extract was calculated as a mass percentage of dry residue (g/100 g extract).

2.5 Determination of steviol glycosides percentages using HPLC

Standard solution (2.2 mg/5 mL) of stevioside was prepared in the mobile phase consisting of mix HPLC grade acetonitrile and bi-distilled water (80:20, v/v). Standard series in the concentration range of 100-1000 µg/mL were obtained from the stock solution. The mobile phase was used as solvent for all HPLC studies. The HPLC analysis conditions were performed by isocratic elution with a flow rate of 0.4 mL/min. All solvents were filtered through a 0.22 µm Millipore filter. Volumes of 5 µL extracts prepared from each sample were directly injected into HPLC then the peak areas at the characteristic wavelength of the steviol glycosides were measured. The UV-VIS detector was set to 210 nm and peak areas were integrated automatically using Agilent software. Separation was carried out using a Supelcosil LC-NH2 or equivalent (length: 15 - 30 cm; inner diameter: 3.9 - 4.6 mm) and flow rate was set to 0.4 mL/min for an isocratic elution at 40 ^{0}C as column temperature. The instrument was calibrated pumping mobile phase through it until a drift-free baseline is obtained. The Agilent software recorded the chromatograms of the sample standard solution.

All the computations concerning the quantitative analysis were performed with external standardization of the measured peak areas. The results were obtained as the mean value of three separate injections. Using the measured peak areas of stevioside from the sample solution and of the standard solution, the percentage of the extracted stevioside was computed as: % stevioside = [Ws/W] x [Aa/As] x 100, where Ws is the mass of stevioside in the standard solution (mg), W is the mass of sample (mg), As and Aa are the peak area of stevioside from standard and sample solutions. The measurements of stevioside from samples of *Stevia rebaudiana* were done according to stevioside standard. At flow rate of 0.4 mL/min the retention time was 1.076 min for stevioside as shown in Figure 2.

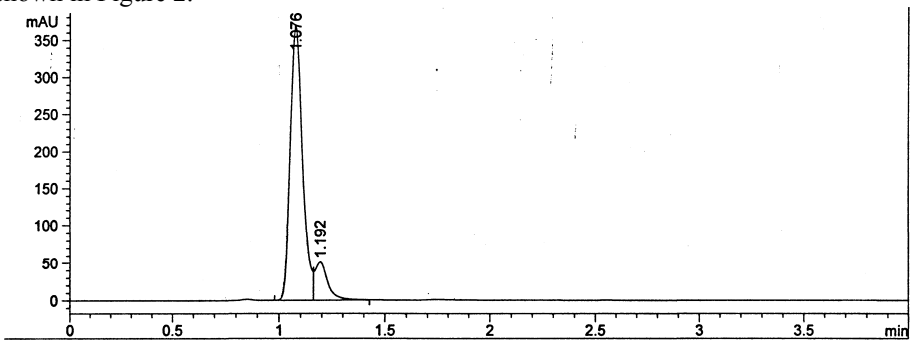

Figure 2. Chromatogram of 2.2 mg/mL standard (Stevioside)

3. Results and Discussions

The HPLC method was applied to quantify the stevioside in the leaves of *Stevia rebaudiana*, the results of different extraction protocols being presented in Tables 1 and 2, and Figures 3 and 4.

3.1 Results obtained for classic extraction

Table 1. Maceration extraction – dry residual and extracted stevioside obtained after 24 hours

	a. Sample weight to solvent volume ratio			*b.* Equivalent diameter dry leaves, mm			*c.* Solvent nature and proportions		
	1/10	1/8	1/5	0.315	2.0	6.3	water	ethanol 55%	ethanol 70%
g d.r. /100 g extract	3.39	4.16	7.07	7.07	3.30	3.17	7.07	4.04	3.28
g stevioside / 100 g extract	0.99	1.05	2.24	2.24	0.97	0.77	2.24	0.98	0.77

a. Influence of the sample weight to solvent volume ratio upon the maceration extraction yields (0.315 mm equivalent diameter dry leaves, distilled water)

b. Influence of the particles size dry leaves on the maceration extraction yields (1/5 sample weight to solvent volume ratio, distilled water)

c. Influence of the solvent nature on the maceration extraction yields (0.315 mm equivalent diameter dry leaves, 1/5 sample weight to solvent volume ratio)

Although not unexpected, the results from Table 1 show a remarkable dependency of extraction upon the granularity of the solid phase and the type of the liquid phase, but

quite normal with respect to the sample weight to solvent volume ratio. As the volume used for extraction increases, the concentration of the extracted species decreases, while their mass could either be the same, or increase too. In the latter case, to whom belongs the actual experiment (data not shown), the extracted species reached the thermodynamic saturation for the lowest volume of the liquid phase used. According to experimental data (Table 2), the dimension of particles plays a key role in extraction of soluble components from *Stevia rebaudiana*, a sharp decrease appearing when passing from sub-millimetre to millimetre range, proving that diffusion inside solid phase is the limiting step. Ethanol seems to inhibit sharply extraction of stevioside from *Stevia rebaudiana*, due, probably, to its higher molecule and lower propensity of having hydrogen bonds with stevioside molecules. From now on, the water will be the extractive agent and the leaves granularity will be 0.315 mm, unless otherwise stated.

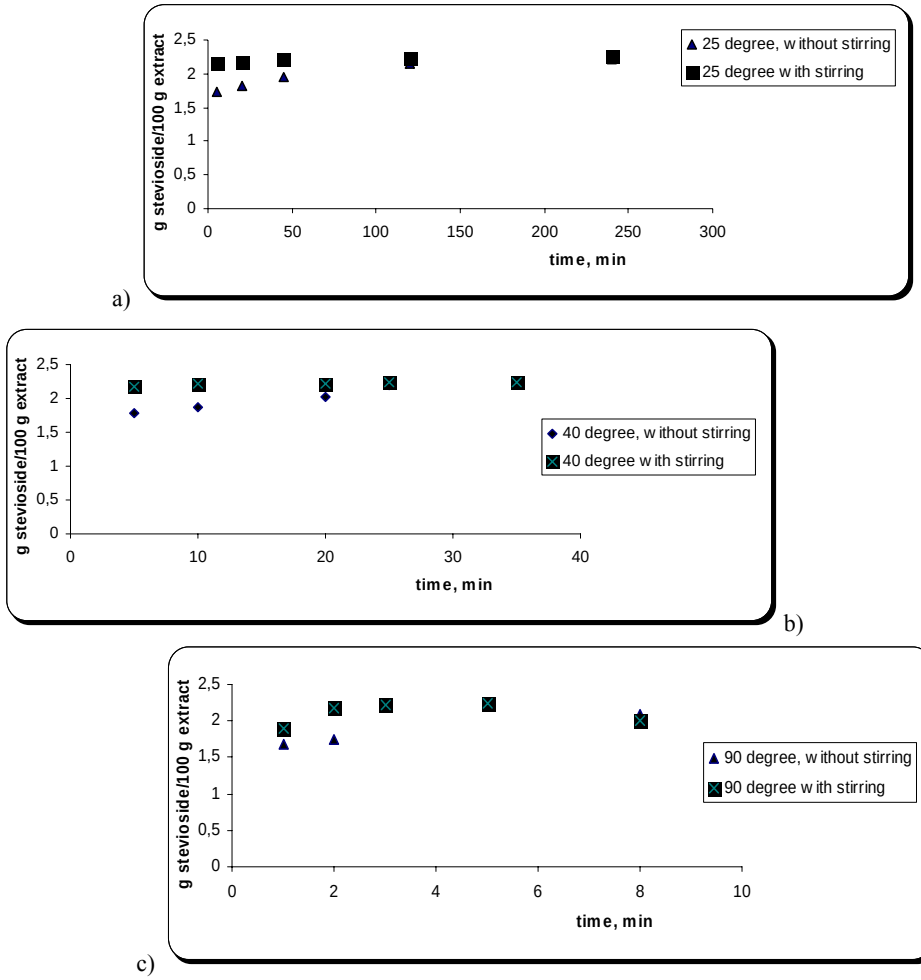

Figure 3. The results for classic extraction; the influence of temperature and stirring upon the extraction yield in time a) 25ºC, b) 40 ºC, c) 90 ºC

When a supplemental kinetic energy is introduced from outside into the liquid phase

through heating, what modifies is the process rate, not the thermodynamic equilibrium, as can be observed from Figure 3, a)-c). If stirring is superimposed (increasing even more the kinetic energy of the liquid phase dissipating mechanical energy), the extraction process becomes even faster, although for the highest temperature its contribution decreases significantly (see, for comparison, the vertical distance between points at the beginning of the process in Figure 3, a-c). The thermal degradation of the valuable compound starts manifesting, as an important downside of increasing the temperature of extraction (Figure 3, c).

3.2 Ultrasound assisted extraction experiments

Table 2. Ultrasound assisted extraction results obtained after 20 min of sonication

	a). Equivalent diameter of dry leaves, mm				*b).* Solvent nature		
	0.315	2.0	6.3	crushed leaves	water	ethanol 55%	ethanol 70%
g d.r./100 g extract	7.08	7.02	7.0	6.97	7.08	7.05	7.03
g stevioside / 100 g extract	2.26	2.25	2.22	2.20	2.26	2.25	2.23

a). Influence of the equivalent diameter of dry leaves upon the ultrasound assisted extraction yields (1/5 sample weight to solvent volume ratio, distilled water)

b). Influence of the solvent nature upon the ultrasound assisted extraction yields (0.315 mm equivalent diameter of dry leaves, 1/5 sample weight to solvent volume ratio)

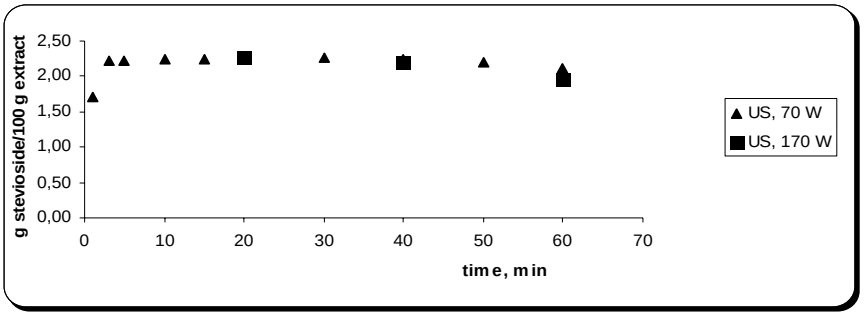

Figure 4. Ultrasound - assisted extraction results. Influence of the output ultrasound power on the extraction yield on time (70 W and 170 W, respectively).

When using ultrasound waves as intensification technique, the extraction rate increases dramatically, the stevioside concentration attaining its maximum value for less than five minutes (see Figure 4 for details). At the same time, neither the granularity of the solid, nor the type of the liquid seem not to count anymore in the economy of the extraction process, as pointed out by the data from Table 2. Even when crushed leaves are used, not only the dry residual is almost the same, but also the stevioside concentration. Also, the presence of ethanol becomes unimportant, the yield in stevioside having the same value as for pure water. This is the effect of the sharp increase of the local turbulences, which increase the mass transfer through diffusion inside the solid and also faster mixing of the liquid, thus maintaining the highest possible driving force. Increasing the

power of the ultrasonic field has no beneficial effect, proving that an optimum ratio of power and liquid, solid or both volumes should exist. The main drawback is the danger of overexposure, when the valuable species ends up being destroyed (see Figure 4, the decrease in the concentration of stevioside for long times), although this could be a threat only for labile species.

3.3. Comparative discussions

The experimental results show that stevioside content of *Stevia rebaudiana* dry leaves is higher than reported in literature. The highest stevioside content was removed from the solid phase by ultrasound assisted extraction, about 10.26 % of dry leaf weight. Compared to maceration, ultrasound assisted extraction increased the productivity more than two hundred times, decreasing the time of completion. With respect to hot extraction, whose performance is by far better than maceration, ultrasound assisted extraction has the main advantage of working at ambient temperatures, thus avoiding the thermal overexposure. Contrary to ultrasound overexposure, the thermal one has a very narrow time window, largely increasing the probability of happening thus needing a rather tight control (see Figure 3, c, where there are only three minutes between optimum and thermal degradation, in Figure 4 this interval is over fifty minutes). Like hot extraction, ultrasound assisted intensification needs special equipment to be functional, which means higher investments, and electricity to produce the ultrasonic waves, which means higher operating costs than maceration. So, a soundly economic analysis should be done, in order to choose the best extraction procedure.

4. Artificial Neural Network modelling

Taking into account the complexity of the ultrasound assisted extraction, given not only by the multitude of the implied parameters, but also from the complex interactions between the mass transfer and the local velocity field heavily affected by the ultrasonic waves, the Artificial Neural Network concept was considered for modelling. An ANN is composed of elements (artificial neurones, organised in layers as the topological structure of the brain, but far less complicate) that perform in a manner similar to the most elementary functions of the biological neurone. The ANN mimics a number of the brain's characteristics: learn from experience, generalise from previous examples and abstract essential characteristics from input containing scattered data, as any self-organizing system. Learning means to present, repeatedly, to an ANN a set of couples of input/output vectors, and to force it to optimize some metric like a sum of squared distances real per desired output. A synthetic neuron (except those of the input layer) processes the sum of the weighted signals received from its dendrites according to its threshold function and outputs the answer through its axon to the following neurons. The threshold function could be of any type of the generalized logistic curve. Matching the ANN output to the real world passes through the attached neuron weights, which modify until the learning criterion is fulfilled. Generalization means the capacity to give a correct answer to a question outside the learning set and relies on ANN capacity of finding out the hidden rules that govern the process, even if, at this time, it can not be mathematically expressed. Anyway, the time consuming step is the learning phase.

The feed-forward ANN used to model the ultrasound assisted extraction has the simplest structure with a single neuron in the hidden layer, three neurons in the input layer (equivalent diameter of dry leaves, sample weight to solvent volume ratio and

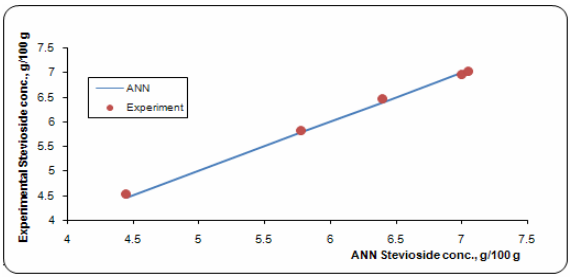

Figure 5. The parity plot of ANN vs. Experimental concentrations of stevioside

output power) and five neurons in the output layer, for the time profile (up to ten minutes) of the stevioside concentrations. This way the ANN has the minimum possible number of weights which should be computed during the learning phase, for which the back-propagation algorithm was chosen. The lack of abundant data prevent us from making a thorough analysis of the best ANN topology, with the present data verifying only the capacity of the given ANN to satisfactorily model the ultrasound assisted extraction. A parity plot of the experimental vs. the learnt profiles is given in Figure 5, proving their good fit.

5. Conclusions

In this study, a HPLC method was developed and applied to quantify the stevioside content of the *Stevia rebaudiana*. Compared with classical extraction methods like maceration and hot extraction, the ultrasound assisted extraction proved to be a simpler but more effective procedure to obtain active compounds from medicinal plants; it works at lower temperatures, avoiding thermal degradation and higher rates, a very important asset for industry. ANN seems to be a good choice for modelling this process.

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6. References

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