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Short communication

On-line monitoring of stevioside sweetener hydrolysis to steviol in acidic aqueous solutions

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ABSTRACT

Stevioside, a potent sweetener obtained from leaves of *Stevia rebaudiana* (Bertoni), is the glycone of steviol. However, despite its natural origin, there has been concern about stevioside toxicity due to hydrolysis to the carcinogenic steviol. To approve it as an additive, the FAO/WHO committee on food additives has required further information about hydrolytic stability of the steviol glycoside in acidic foods and beverages. In this study, aqueous solutions of stevioside at different pH values were monitored in real time by direct infusion ESI-MS. Owing to the high speed and sensitivity of ESI-MS monitoring, fast hydrolysis of the stevioside molecule to steviol in aqueous acidic solutions was observed, particularly in acidic juices.

1. Introduction

The stevioside is a natural sweetener extracted from the leaves of *Stevia rebaudiana* (Bertoni). It is commercialised as *Stevia* and its attractive features is the natural origin, as compared to other major artificial sweeteners (aspartame and cyclamate for instance) as well as its high sweetening potency, which is 250–300 times greater than that of sucrose (Kinghorn, Wu, & Soejarto, 2001). The *stevioside* molecule **1** (Fig. 1) is comprised of a glycone (sugars) attached to the steviol moiety. The class of Stevia-related sweeteners has been indicated to benefit the glucose metabolism (Jeppesen, Gregersen, Alstrup, & Hermansen, 2002) and renal function (Hsieh et al., 2003). Despite its natural origin and possible benefits, there have been serious concerns about its safety; hence, the toxicity, carcinogenicity and genotoxicity of stevioside have been investigated. These studies have been conducted mainly in Japan, where Stevia is approved as a food additive (Aze et al., 1991; Matsui, Sofuni, & Nohmi, 1996; Matsui et al., 1996; Pezzuto, Compadre, Swanson, Nanayakkara, & Kinghorn, 1985; Toskulkao, Chaturat, Temcharoen, & Glinsukon, 1997; Xili et al., 1992). The results have often suggested that stevioside has no serious toxicity to mammals. Recently, however, an *in vitro* study of the metabolism of several glycosidic sweeteners showed that Stevia-related compounds are degraded to steviol **2** (Fig. 1) by human faecal homogenates, and no apparent inter-species differences in the intestinal metabolism between rats and humans of Stevia-related compounds were observed (Koyama et al., 2003). Since steviol is highly lipophilic, it has been postulated that it will be absorbed into the systemic circulation (Wingard et al., 1980). Steviol has also been known to be mutagenic after metabolic activation in the mutation assay using *S. typhi* TM677 (Pezzuto et al., 1985), and a possible decrease of the fertility of male rats was also suggested (Melis, 1999). This apparent toxicity led Australia and Canada, for instance, to approve Stevia only as a food supplement, but not as a food additive. These studies provide therefore conflicting conclusions and insufficient toxicological information about the safety of steviol. Therefore, the concerns about the safety use of the natural stevioside sweetener still remain (WHO, 1999). Lack of critical scientific reports on stevioside and their discrepancies about the toxicological effects of its aglycone steviol led the European Commission in 2000 to refuse to accept Stevia as a food or drug additive (FAO/WHO, 2004).

Normally, stevioside and steviol have been analysed by HPLC with ultraviolet detectors (Hutapea, Toskulkao, Wilairat, & Buddhasukh, 1999; Koyama et al., 2003). Herein we applied direct infusion ESI(+)-MS for the on-line monitoring of stevioside hydrolysis. ESI(+)-MS has been used as an interesting "ion-fishing" technique, since it is able to gently transfer with high speed and sensitivity either positive or negative ions (even transient species) directly from solutions to the gas phase (de la Mora et al., 2000). Due to these outstanding features, ESI-MS (and its tandem version ESI-MS/MS) is rapidly becoming a major tool in chemistry and biochemistry for the fast screening of reaction intermediates in solution (Santos, 2008, 2010; Santos, Knaack, & Metzger, 2005) with clear structural characterisation (Wu, Rodgers, & Marshall, 2004).

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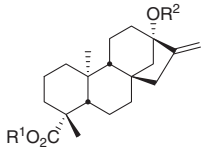

1, Stevioside, R^1 = β-Glc; R^2 = β-Glc-β-Glc (2-1)
2, Steviol, R^1= R^2= H

Fig. 1. Structures of stevioside (**1**) and the hydrolysed derivative steviol (**2**).

2. Experimental section

2.1. Sample preparation

The Stevia sweetener (Steviafarma) samples (500 μl) were diluted in a flask with a 1:1 solution of H_2O:MeOH (Merck, Darmstadt, Germany) to a final volume of 1.0 ml. The screening of degradation of Stevia in different pH was performed by acidification of solutions containing Stevia adjusted with HCl (Merck, Darmstadt, Germany) aqueous solutions. pH values were monitored by commercial (Merck, Darmstadt, Germany) indicator strips. Orange, passion fruit, lemon juices, and coffee were analysed by direct injection of the samples after addition of the sweetener.

2.2. ESI-MS monitoring

The samples were directly infused at a flow rate of 5.0 μl min^{-1} using a syringe pump. ESI-MS and ESI-MS/MS in the positive ion mode were acquired using a Waters Q-TOF Micro instrument with 5000 mass resolution in the TOF mass analyser. Typical operating conditions were 3.5 kV capillary voltage, 35 V cone voltage, and desolvation gas temperature of 100 °C. ESI-MS/MS were collected by causing collision-induced dissociation (CID) of the mass-selected protonated molecules using argon as the buffer gas and collision energies from 18 to 25 eV. Ion-selection was performed by Q1, and collisions were performed in the rf-only hexapole collision cell, followed by mass analysis of product-ions by the high-resolution orthogonal-reflectron TOF analyser. ESI-MS were acquired over a *m/z* range of 50–1200. HPLC methanol grade and HCl were purchased from Merck (Darmstadt, Germany) and used without further treatment.

3. Results and discussion

3.1. Hydrolysis monitoring

As an initial test, the ESI-MS screening of solutions containing the strevioside **1** was carried out by adjusting the cone and ion-source voltages. This preliminary tuning was necessary to minimise or likely eliminate possible in-source CID of protonated **1** to the aglycone species **2**–**4** (Fig. 2). Fig. 3a shows the ESI(+)-MS of stevioside H_2O:MeOH (1:1 v/v) solutions at its natural pH 4. Note that **1** is detected mainly by its potassium adduct [**1** + K] of *m/z* 843. Then, to test the source lability of gaseous [**1** + K] the voltages of the ion-source (between 3000 and 4000 V) as well as the cone (15–80 eV) were varied. However, [**1** + K] fail to dissociate at any significant extent (Fig. 3a). Next, seven different aliquots of

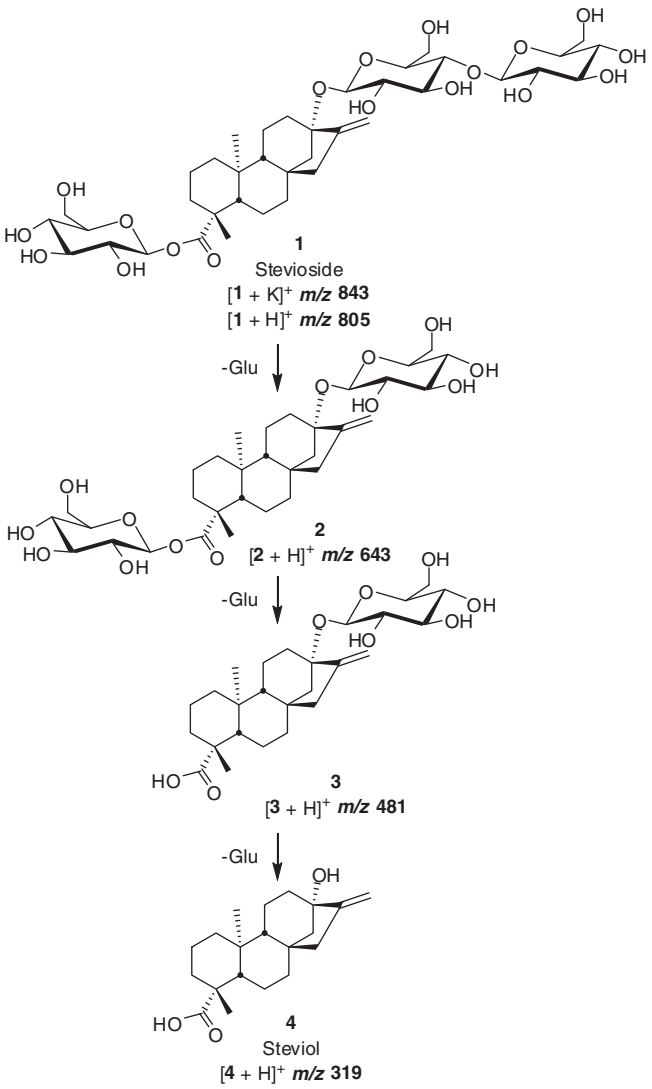

Fig. 2. Proposed sequence for stevioside hydrolysis in acidic media according to ESI-MS(/MS) on-line monitoring.

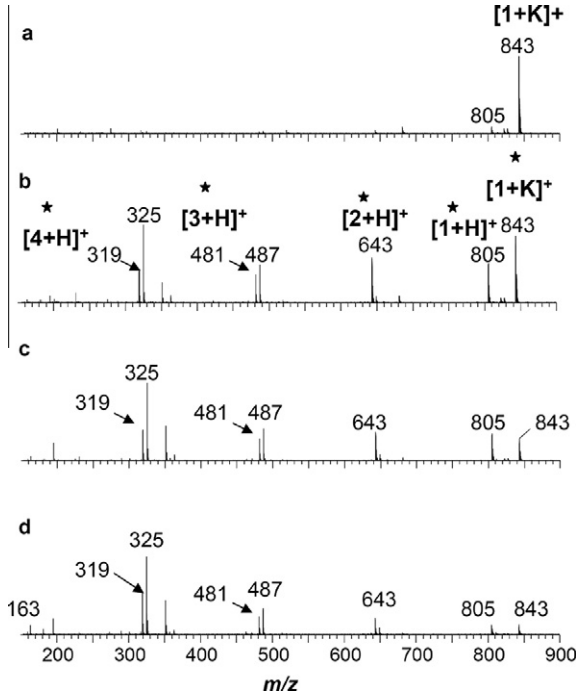

Fig. 3. ESI(+)-MS of Stevioside after 30 s in different acidic medium: (a) pH 4, (b) pH 3, (c) pH 2, and (d) pH 1.

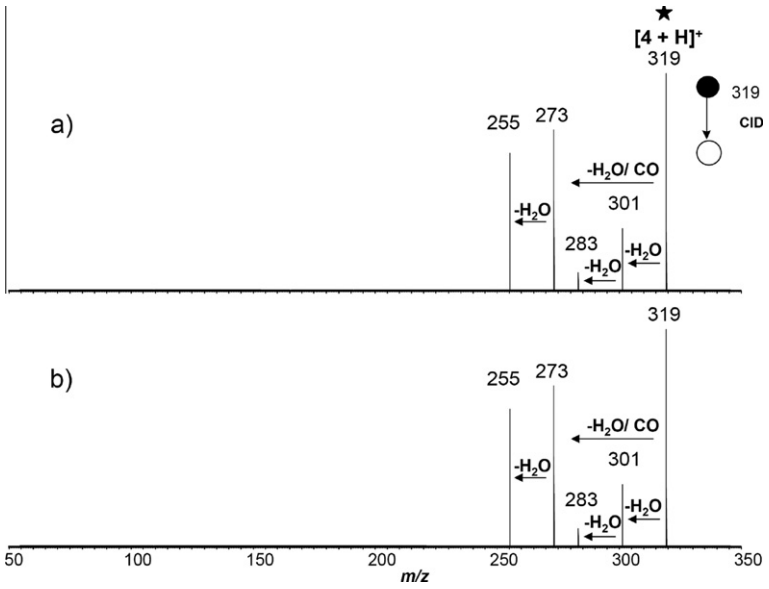

Fig. 4. ESI(+)-MS/MS of protonated steviol [**4** + H]⁺: (a) aqueous solution; (b) orange juice.

aqueous solutions of *Stevia* at different pHs (adjusted by the addition of HCl) were analysed by ESI(+)-MS after dilution in water:methanol (1:1). The stevioside **1** and its aglycones **2**–**4** should be detected by ESI(+)-MS either as its protonated [M + H] or cationized forms [M + Na] or [M + K] (Fig. 2). Fig. 3a–d shows therefore the ESI(+)-MS of stevioside solutions at different pH after 30 s of sweetener addition. As already discussed, [**1** + K] of *m/z* 843 is the main species detected at pH 4 (Fig. 3a). At pH 3 up to 1, both [**1** + H] of *m/z* 805 and [M + K] of *m/z* 843 are detected with decreasing abundances, whereas the protonated forms of hydrolysis products: [**2** + H] of *m/z* 643, [**3** + H] of *m/z* 481 and [**4** + H] of *m/z* 319 are detected with increasing abundances (Fig. 3b–d).

To assure that the ion of *m/z* 319 is in fact protonated steviol [**4** + H], a solution of steviol was prepared from a commercial standard and its ESI(+)-MS/MS acquired (Fig. 4), showing the same dissociation pattern as that sampled from the hydrolysis experiment (not shown). Furthermore, HPLC-UV-ESI(+)-MS analysis were performed by using a gradient solvent system consisting of acetonitrile and 10 mM ammonium acetate at a flow rate of 0.8 ml/min. The percentage of acetonitrile was increased from 30% to 85% over 40 min. After 40 min, the column was re-equilibrated with the initial mobile phase for 10 min. ESI-MS full scan spectra were obtained from stevioside incubated with HCl for 30 s, showing the ions at *m/z* 805 (R_t = 8.5 min), *m/z* 643 (R_t = 11.9 min), *m/z* 481 (R_t = 15.4 min) and *m/z* 319 (R_t = 20.1 min) that were identified on the ion chromatograms throughout pH 1. The R_t and fragmentation patterns observed in the positive ESI ion mode for these ions were in accordance with the standards of stevioside, **2**, **3**, and steviol **4**.

Next, the stability of **1** in beverages that are commonly sweetened with stevioside **1** were also evaluated (coffee as well as orange, lemon and passion fruit juices). For that, 500 μl of an 12% m/V aqueous solution of **1** were added to 10 ml of the beverage and the ESI(+)-MS acquired after 30 s of mixing. For coffee (pH around 5–6), no ions related to stevioside hydrolysis to steviol was observed by adding the sweetener to hot coffee (Fig. 5a). However, for orange juice (pH around 2.5), hydrolysis to **4** was clearly indicated by the detection of [**4** + H] of *m/z* 319, and by its

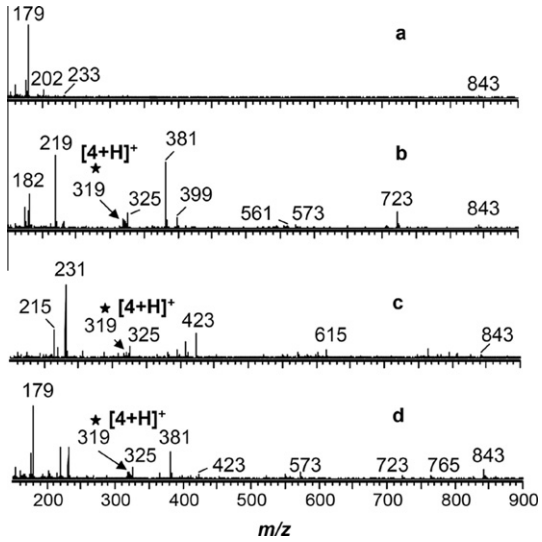

Fig. 5. ESI(+)-MS monitoring of beverages sweeten with Stevia: (a) coffee, (b) orange, (c) lemon, (d) passion fruit.

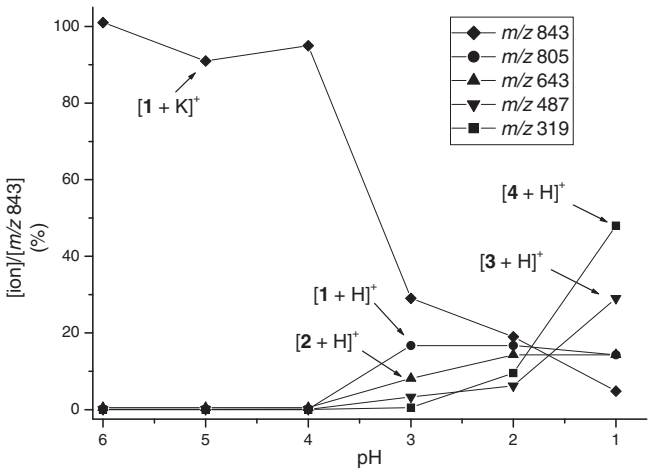

Fig. 6. Hydrolysis of **1**–**2** via **3** and **4** in water as a function of pH using data from the ESI(+)-MS monitoring.

ESI(+)-MS/MS, which was identical to that of standard **4** (Fig. 4). Similar behaviour was observed for lemon (pH 2.0, Fig. 5c) and passion fruit (pH 2.0, Fig. 5d) juices. Hydrolysis of **1**–**2** via **3** and **4** in water was therefore plotted as a function of pH using data from the ESI(+)-MS monitoring (Fig. 6). Similar plots were obtained from the acidic beverages.

4. Conclusion

Direct infusion ESI(+)-MS, due to its high speed and sensitivity and direct on-line monitoring ability, has confirmed acid hydrolysis of stevioside **1** to steviol **4** in aqueous solutions as well as in acidic beverages such as coffee and fruit juices. The ESI(+)-MS data indicates that **1** hydrolyses fast and quite extensively to **4** via intermediates **2** and **3**, and that this reaction is very fast (**4** is detected in less than 30 s) particularly under low pH. Concerns about the safety of Stevia-related sweeteners should now reside on determining whether or not steviol is a safe molecule for humans in the highly acidic stomach, and the safety amounts for daily consumption.

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