Determination of Caffeine and Isohumulones in Saliva by HPLC Analysis

Shuya Tanimura

Sensory Evaluation Section, Research and Development Division, Kirin Brewery Company, Ltd., 6-26-1 Jingumae, Shibuyaku, Tokyo 150, Japan

A high-performance liquid chromatographic (HPLC) procedure was applied to investigate the occurrence of two widely consumed bitter substances in human saliva: caffeine (CA) and iso-alpha-acids (IAA, beer bittering agents, so-called isohumulones, primarily isohumulone, isocohumulone, and isoadhumulone). An HPLC analysis with pre-extraction by disposable cartridge was successfully applied to detect CA. CA was detected in the saliva of most CA users, but the amount of CA found in saliva was not related to individual bitter taste sensitivity. IAA were not detected in saliva of beer users by HPLC analysis.

Keywords: *Caffeine; isohumulones; saliva; HPLC*

INTRODUCTION

From the standpoint of evaluating bitterness in food, nonusers of food containing bitter compounds [caffeine (CA) for coffee and tea, iso-alpha-acids (IAA) for beer, etc.] are assumed to be more sensitive to the bitterness of these items than users of bitter foods. Thus, their heightened sensitivity may play a causal role in their low level of intake (Tanimura and Mattes, 1993).

CA is a commonly consumed bitter substance that reliably appears in saliva shortly after ingestion (Zylber-Katz et al., 1984). We could not confirm the effect of acute and chronic CA ingestion to bitter taste sensitivity through dosing tests of non or slight CA users (less than 100 mg/day; Mela et al., 1992). In this research, the effect of habitual CA ingestion was evaluated and bitter taste sensitivity and salivary level of CA were compared under subjects' ordinary usage of the compound.

Bitterness is a desirable characteristic in beer and is achieved by adding hops to wort in beer making. The important bittering components are alpha-acids. During the wort boiling process befor fermentation, alpha-acids are transformed into iso-alpha-acids (IAA, more bitter than alpha-acids and the so-called isohumulones, primarily isohumulone, isocohumulone, and isoadhumulone; Palamand and Aldenhoff, 1973) which give beer its characteristically bitter taste. There is little information on the transfer of IAA to humans, compared to organoleptic and chemical work on hop-derived bitterness (Buckee, 1985; Verzele et al., 1981; Saag, 1988; and others). There was no report related to the determination of the salivary level of IAA except the author's own study (Tanimura and Mattes, 1993). In that study using an acute dosing test, IAA were not detected in saliva.

HPLC is one of the most recent techniques to be used in the field of food analysis (Macrae, 1988). In this research a reversed-phase HPLC technique was applied to determine CA (Hartley et al., 1985) and IAA (Verzele et al., 1981) levels in human saliva.

MATERIALS AND METHODS

Chemical Reagents. CA, hydroxytheophylline (internal standard for CA analysis), and phenanthrene (internal standard for IAA analysis) were purchased from Sigma Chemical Co. (St. Louis, MO). Purified IAA were kindly supplied by Kalsec, Inc. (Isolone product name; Kalamazoo, MI).

Chromatographic Equipment. For CA analysis, an M45 solvent delivery system (Waters Associates, Inc.) was used with a UVIDEC-100-III detector (Japan Spectroscopic Co.). For IAA analysis, an M660 solvent programer with two M6000A pumps was used for gradient elution with the same UVIDEC-100-III detector.

Chromatographic Conditions. For CA analysis, an analytical column packed with Perkin-Elmer Pecospher (5 μm, C_{18} ODS, 4.6 × 150 mm) was used according to the method of Hartley et al. (1985). The peak area was determined by UV absorbance at 273 nm. For IAA analysis, the method of Verzele et al. (1981) was modified for the procedure of gradient elution. The mobile phase for gradient elution was started from methanol–water (1:1) to 100% methanol (all solvents contain 0.5% phosphoric acid) for 30 min at 2.0 mL/min. IAA were detected by UV at 280 nm. The same precolumn as that used for CA analysis was used.

Recovery Studies Using Saliva. In the case of pre-extraction of CA from saliva, a disposable solid-phase cartridge [Bond-Elute column packed with C_{18} bonded silica by Analytichem International, Harbor City, CA; described by Reid and Good (1982)] was used. A recovery test using the cartridge was conducted by adding CA solution to the saliva of one subject in which no CA was detected. For extraction of CA by the cartridge, 0.2 mL of CA solution in the range 0.96–7.66 μg/mL was added on it and washed with 2 mL of water. Then the CA was eluted by 0.4 mL of methanol, and 0.1 mL of hydroxytheophylline (10 μg/mL) was added to it. Then the combined eluant and standard solution were evaporated to dryness and redissolved in 0.5 mL of methanol. Ten microliters of each solution was analyzed for determining the HPLC peak area of CA. In the case of a recovery test for IAA, IAA were added to the saliva of one subject in which no IAA were detected (adding range: 0.1–2.0 μg/mL).

Saliva Collection and Bitter Taste Sensitivity Measure. The method of Navazesh and Christensen (1982) was used for the collection of stimulated whole saliva [described by Tanimura and Mattes (1993); based on the study of Navazesh and Christensen (1982)]. In CA analysis, extraction from saliva was achieved using a Bond-Elute cartridge. For IAA, saliva was centrifuged at 1500 rpm for 15 min and the supernatant was used directly for the analysis. Individual bitter taste sensitivity to CA and IAA was determined by detection threshold (Tanimura and Mattes, 1993). Subjects refrained from eating and drinking for at least 2 h before testing. The threshold testing solutions consisted of CA in deionized water at concentrations ranging from 7.9×10^{-3} to 3.15×10^{-5} M by one-fifth log dilution steps. In the case of IAA, the concentration range of the solution was from 50 to

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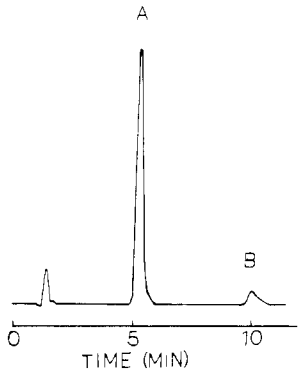

Figure 1. Detection of caffeine in human saliva: peak A, internal standard (hydroxytheophylline); peak B, caffeine.

Table 1. Recovery of Caffeine from Saliva by Using Extraction Column[a]

caffeine concn level (μg/mL)	recovery of added caffeine (%)	caffeine concn level (μg/mL)	recovery of added caffeine (%)
0.96	103	3.83	98.1
1.92	102	7.66	94.3

[a] Extraction column: Bond-Elute column packed with C_{18} bonded silica by Analytichem International.

Table 2. Salivary Level of Caffeine in Nonusers

subject no.	sex	test no.	caffeine level (μg/mL)
1	female	1	0
		2	0
2	male	1	0
		2	0
3	female	1	0
		2	0
4	female	1	0
		2	0.49
5	male	1	0
		2	0
6	male	1	0
		2	0
7	female	1	0
		2	0

0.20 μg/mL by half-dilution steps. Detection threshold and saliva collection for HPLC analysis were assessed on two occasions separated by at least 2 days.

RESULTS

Comparison of Caffeine Threshold with Salivary Caffeine. CA was detected in human saliva (Figure 1). The result of CA recovery studies is shown in Table 1. Seven nonusers (three males and four females; mean age, 25.7 ± 3.9 years) were tested for salivary CA levels in two separate testing sessions (shown in Table 2). Fifteen users (eight males and seven females; mean age, 27.9 ± 6.1 years) were also tested for the same measure (shown in Table 3).

CA thresholds of the two groups (4.1×10^{-4} M in nonusers and 1.2×10^{-3} M in users; $p < 0.01$ by t-test) were significantly different. Salivary CA was not detected in nonusers except in one of the two test sessions of one subject, but it was detected in 11 of the 15 users. Thus, the salivary CA level in users was significantly higher than that in nonusers (mean salivary CA, 0.19 μg/mL in users and 0.03 μg/mL in nonusers, $p < 0.01$ by t-test). Among CA users, the CA threshold has no significant correlation to salivary CA (correlation coefficient, $r = 0.329$, $p > 0.05$).

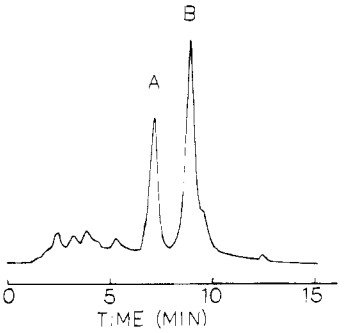

Figure 2. Detection of iso-alpha-acids in wort extraction: peak A, isocohumulone; peak B, isohumulone and isoadhumulone.

Table 3. Salivary Level of Caffeine in Users

subject no.	sex	test no.	caffeine level (μg/mL)
1	male	1	0.28
		2	0.34
2	female	1	0
		2	0
3	female	1	0.38
		2	0.38
4	female	1	0
		2	0.09
5	female	1	1.07
		2	0.57
6	female	1	0
		2	0
7	female	1	0
		2	0
8	male	1	0.20
		2	0
9	female	1	0.25
		2	0.46
10	male	1	0
		2	0
11	male	1	0.13
		2	0
12	male	1	0
		2	0.15
13	male	1	0.53
		2	0
14	male	1	0.25
		2	0
15	male	1	0.23
		2	0.27

Nonappearance of Iso-Alpha-Acids in Saliva. IAA were successfully detected through wort extraction (Figure 2). The recovery of IAA from saliva was 98% (mean of both test sessions in 0.1, 0.5, 1.0, and 2.0 μg/mL levels). Eight beer users (six males and two females; beer consumption level, 885 ± 266 mL/day) were tested for their salivary IAA levels, but IAA were not detected in any of the subjects (detection limit, 0.1 μg/mL). The mean IAA threshold of the eight users was 2.6 μg/mL (variance, 0.98 μg/mL).

DISCUSSION

Salivary CA was detected in the saliva of most CA users. It is considered that salivary CA closely parallels

plasma levels (Zylber-Katz et al., 1984). Individuals who frequently consume CA are likely to maintain detectable levels of CA in their saliva at almost all times. Although dietary conditions influence individual taste sensitivity via saliva, the sensitivity to CA may be determined by inner factors (genetic, etc.; Tanimura et al., 1994). Diabetic patients (genetically determined) have elevated taste thresholds, especially to quinine (Settle, 1991). It has been proposed that functionally significant relationships exist between salivary level and gustatory perception of sodium chloride (Morino and Langford, 1978). In this study, salivary CA concentration has only a weak correlation to the taste threshold of CA ($p > 0.05$). Besides taste sensitivity to salt, bitter taste sensitivity is assumed to be determined by an individual inner factor (genetic).

Compared to the poor reproducibility of organoleptic determination of bitterness in foods, HPLC permits the exact quantitative determination of each bitter-tasting compound (Macrae, 1988). HPLC methods have the advantage of sensitivity and improved separation (Reid and Good, 1982). For studying the appearance of bitter substances in the saliva of users, HPLC has been utilized to perform a rapid and accurate analysis. In this research it was confirmed that HPLC was successfully applied to detect CA in the saliva of CA users under ordinary diet conditions.

The detection threshold of IAA is lower than the CA detection threshold by about 2 log orders; the bitterness of IAA is stronger than that of CA (Tanimura and Mattes, 1993). In this test, IAA were not detected in the saliva of beer users. The possibility exists that the detection of IAA in saliva is more difficult than that of CA, because of the low level of IAA in it. The development of a more sensitive method may be required to detect the bitter substances.

Considering the results of this research (beer consumption level, about 800 mL/day) and of the earlier acute dosing test of IAA by the author (approximate IAA intake is equal to about that from 2000 mL of beer; Tanimura and Mattes, 1993), it is assumed that IAA is not transferred to saliva in ordinary beer intake.

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Received for review August 3, 1993. Revised manuscript received April 15, 1994. Accepted May 9, 1994.[⊗]

[⊗] Abstract published in *Advance ACS Abstracts,* July 1, 1994.