Title:

An acetone bio-sniffer (gas phase biosensor) enabling assessment of lipid metabolism from exhaled breath

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24 acetone; biosensor; fiber-optic; secondary alcohol dehydrogenase;

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26

27 **Abstract**

28 Several volatile organic compounds (VOCs) are released from human
29 breath or skin. Like chemical substances in blood or urine, some of these vapors
30 can provide valuable information regarding the state of the human body. A highly
31 sensitive acetone biochemical gas sensor (bio-sniffer) based on the
32 consumption of nicotinamide adenine dinucleotide (NADH), was developed and
33 used to measure exhaled breath acetone concentration, and how this changed
34 in response to lipid metabolism.

35 A fiber-optic biochemical gas sensing system was constructed with an
36 ultraviolet-light emitting diode (UV-LED) excitation system ($\lambda=340$ nm), a
37 photomultiplier tube (PMT) and an optical fiber probe. NADH-dependent
38 secondary alcohol dehydrogenase (S-ADH) was immobilized on the optical fiber
39 probe. Measurement of acetone concentration was carried out by immersing the
40 sensor probe in phosphate buffer solution containing NADH. NADH is consumed
41 by the enzymatic reaction of S-ADH, and the consumption is proportional to the
42 concentration of acetone vapor. The change of fluorescent emitted from NADH

43 is analyzed by the PMT. Hence, fluorescence intensity decreased as the acetone
44 concentration increased. The relationship between fluorescence intensity and
45 acetone concentration was identified from 20 ppb to 5300 ppb. This interval
46 included the concentration of acetone vapor in the breath of healthy people and
47 those suffering from disorders of carbohydrate metabolism. Finally, the acetone
48 bio-sniffer was used to measure breath acetone during an exercise stress test
49 on an ergometer after a period of fasting. The concentration of acetone in breath
50 was shown to significantly increase after exercise. This biosensor allows rapid,
51 highly sensitive and selective measurement of lipid metabolism.

52

53 Introduction

54 Blood tests are common diagnostic tools enabling acquisition of valuable
55 information regarding the metabolic function of the human body. However, they
56 are invasive, potentially causing physical and psychological distress. In recent
57 years, breath analysis as a tool for diagnosis and physiological assessment is
58 evolving rapidly on a global scale (Hunter and Dweik 2008; Manolis 1983).

59 Exhaled breath condensate (EBC) and volatile organic compounds (VOCs) are
60 two major topics in breath analysis (Kuban and Foret 2013). EBC is the liquid
61 phase of the exhaled breath. It predominantly consists of water vapor and
62 aerosol particles from the lower respiratory tract (Di Natale et al. 2014). EBC has
63 been used increasingly as a sample to detect viruses or bacteria (e.g., detection
64 of influenza viruses in EBC samples using silicon nanowire sensor devices)
65 (Shen et al. 2012). There are hundreds of VOCs in exhaled breath, some of
66 which are also present in the bloodstream (Amann et al. 2014). Hence breath
67 analysis offers a unique opportunity for non-invasive measurement (Bloemen et
68 al. 2009; Riess et al. 2010; Yeh et al. 2008).

69 Acetone is a typical VOC in exhaled breath. It is affected by fasting, exercise and
70 diabetes mellitus (Blaikie et al. 2014; Mork and Johanson 2006; Schubert et al.
71 2012; Wang and Wang 2013). Biochemical pathways of acetone production are
72 well understood. Acetone is metabolized by 2 pathways: Decarboxylation of
73 acetoacetate or dehydrogenation of 2-propanol. Acetoacetate is generated by
74 dextrose metabolism and lipolysis, and is a major source of acetone in the
75 human body (Musa-Veloso et al. 2002; Musa-Veloso et al. 2006). Acetone
76 concentrations in exhaled breath have previously been shown to correlate
77 strongly with acetone concentrations in blood, as well as with other ketones such
78 as beta-hydroxybutyrate (Blaikie et al. 2014; Tanda et al. 2014). A correlation
79 between acetone concentration in exhaled breath and blood glucose
80 concentration has also been reported (Righettoni et al. 2013). The acetone
81 concentration of exhaled breath in healthy people ranges from 200 to 900 ppb in
82 previous research (Righettoni and Tricoli 2011; Toyooka et al. 2013). Increases
83 in breath acetone concentration are usually a sign that cells lack insulin or are
84 unable to effectively use available insulin; this occurs in diabetes. The breath

acetone concentrations of diabetic patients are higher than those of healthy people, and can exceed 900 ppb (Rydosz 2014; Sun et al. 2015). For this reason, acetone in exhaled breath is a potential biomarker for non-invasive diagnosis of diabetes.

There are several methods for determining acetone concentration. Low concentrations of acetone can be quantified by gas chromatography with mass spectrometry (GC/MS) (Deng et al. 2004; Fan et al. 2014; Phillips 1997; Qin et al. 1997). Though these methods are accurate, they have various disadvantages: They are expensive, time-consuming, operator-dependent and they are also unable to provide continuous measurements. Proton Transfer Reaction Mass Spectrometry (PTR-MS) is a continuous detection method for analysis of exhaled breath samples using gas phase hydronium ions as ion source reagents (Schwarz et al. 2009a; Schwarz et al. 2009b). However, species identification and quantification can be complicated by the presence of other compounds and fragments with the same mass to charge ratio. Nevertheless, a cell-based gas biosensor for detecting acetone vapor by direct exposure of gaseous substances

has been recently designed and validated (Bohrn et al. 2013).

Due to the shortcomings of the above mentioned methods, a rapid, convenient, non-invasive and highly selective method for gaseous acetone detection is strongly required. This might be achieved using an enzyme-based biosensor. Several enzymes such as acetone carboxylase catalyze the oxidation of acetone. One such enzyme, nicotinamide adenine dinucleotide (NADH)-dependent dehydrogenase, is suitable for use in biosensors. Secondary alcohol dehydrogenase (S-ADH) catalyzes the reduction of acetone in the presence of NADH, which acts as an electron donor. 2-propanol and oxidized nicotinamide adenine dinucleotide (NAD^+) are produced through the following enzymatic reaction:

Research regarding NADH-dependent amperometric biosensors has already been undertaken (Sluis and Ensign 1997; Sutak et al. 2012; Yamazaki et

al. 2001). Although this type of sensor is superior in terms of sampling volume, structure and measurement cost, there are several disadvantages to be considered. Most of the electro-active biomolecules such as catecholamines, ascorbic acid and uric acid are oxidized at an electrical potential similar to that of NADH oxidation. In addition, direct oxidation of NADH at bare electrodes results in surface contamination of the electrodes (fouling) due to the adsorption of oxidized products of NADH, resulting in poor sensitivity (Musameh et al. 2002; Nowall and Kuhr 1995). Optical methods are also commonly used for NADH detection. It is known that NADH can be excited by 340 nm ultraviolet (UV) radiation and it has a fluorescent emission peak at 490 nm, while NAD^+ has no fluorescent emission or optical properties.

We have previously reported a fiber-optic NADH sensor (Kudo et al. 2010), which uses a UV-light emitting diode (UV-LED) excitation system and a primary alcohol dehydrogenase (ADH) immobilized membrane for measuring the concentration of ethanol in gaseous samples. Fluorescence intensity increases as NADH is produced through an enzymatic reaction.

Based on this ethanol biosensor, a rapid, compact and highly selective biosensor for acetone vapor was developed using NADH-dependent S-ADH. The biosensor consists of a high-intensity UV-LED excitation system, a photomultiplier tube (PMT) and an optical fiber probe with a S-ADH immobilized membrane. The UV-LED excitation system has a simple and compact structure, due to its low power consumption and low heat generation. LEDs also provide advantages in safety and controllability compared with UV lamps.

Hence, a novel bio-sniffer for continuous monitoring of gaseous acetone by the reverse catalytic reaction of S-ADH was developed. When S-ADH catalyzes the reduction of acetone in the presence of NADH, which acts as an electron donor, 2-propanol and oxidized NAD^+ are produced. This study aims to describe the biosensor design, characteristics and validation through analysis of breath acetone concentration in test subjects at rest and during exercise according to lipid metabolism.

148

149 **2. Experimental Methods**

150 **2.1 Reagents and chemicals**

151 NADH-dependent secondary alcohol dehydrogenase (EC. 1.1.1.x, 1 unit
152 mg^{-1} , E001) originating from yeast, was purchased from Daicel Chiral
153 Technologies, Japan. NADH (the reduced form) was from Oriental Yeast, Japan.
154 Hydrophilic PTFE (H-PTFE, porosity: 80%, pore size: 0.2 μm , JGWP14225), an
155 immobilization enzyme carrier, was from Millipore, USA. 2-methacryloyloxyethyl
156 phosphorylcholine copolymerized with 2-ethylhexyl methacrylate (PMEH) was
157 synthesized according to methods used in previous research (Kudo et al. 2008).
158 pH 7.0, 0.1 M phosphate buffer solution (PB) was prepared by gradually adding
159 0.1 M dipotassium hydrogen phosphate (K_2HPO_4 , 98.0%, 164-04315, Wako
160 Pure Chemical Industries, Japan) to 0.1 M potassium dihydrogen phosphate
161 (KH_2PO_4 , 98%, 160-04275, Wako Pure Chemical Industries, Japan) to obtain
162 the specified pH value. All solutions were prepared using deionized distilled
163 water obtained from a Milli-Q purification system (Millipore, USA). A 50 μM

NADH solution was prepared using the PB (pH7.0, 0.1 M), to act as circulating buffer. A standard acetone vapor was prepared using a standard gas generator (PD-1B-2, GASTEC corporation, Japan).

2.2 Construction of the fiber optic S-ADH bio-sniffer

The acetone bio-sniffer consisted of an excitation source, a photodetector and an optode that produces NAD^+ from acetone. The excitation system utilized a UV-LED (UVTOP[®] BL335, Sensor Electronic Technology Inc., USA) with a UV-LED power supply circuit (GS200, Yokogawa Electric Corporation, Japan). A PMT (C9692, Hamamatsu corporation, Japan) was used as the photodetector. These components were connected to separated ends of a bifurcated optical fiber assembly (core size $600 \pm 10 \mu\text{m}$, BIF600-UV/VIS, Ocean Optics Inc. USA). An optical fiber probe (F1000-900) was connected to the common end of the optical fiber assembly. In order to reduce the background signal, two band-pass filters ($\lambda=340 \pm 10 \text{ nm}$ and $\lambda=490 \pm 10 \text{ nm}$, BPF, Asahi Spectra, Japan) were used in excitation and detection, respectively.

The S-ADH sensitive optode was fabricated by attaching a flow-cell with an S-ADH immobilized membrane to the end of the optical fiber probe. The flow-cell had a cylindrical structure consisting of a silicone tube ($\Phi 2$ mm) and a PMMA cell ($\Phi 4$ mm), forming a socket for the optical fiber probe. Micro flow-channels supplying NADH-containing PB, were formed on the boundary between the silicone and PMMA, depicted in Figure 1. The S-ADH membrane was attached onto the end of the flow-cell as a sensing diaphragm. S-ADH (0.5 unit cm^{-2}) and PMEHS solution (10%, $25 \text{ } \mu\text{l cm}^{-2}$) was mixed and spread carefully onto the H-PTFE membrane filter. The membrane was cured in a refrigerator for 3 hours at 4°C . The S-ADH immobilized membrane was then cut into 2×2 cm and fixed onto the flow-cell with a silicone O-ring.

2.3 Characterization of the fiber optic S-ADH bio-sniffer

Figure 1 shows the experimental setup for monitoring of standard acetone vapor. In this system, the acetone vapor was supplied from the standard gas generator. The flow-rate was controlled using a mass flow controller with a

needle-bulb regulator (Type: RK1200, Koflok, Tokyo, Japan). NADH-containing PB was circulated into the flow-cell by an intelligent pump (INTELLIGENT PUMP, FLOM Co. Ltd., Japan). S-ADH loses its specific activity if chemical conditions are not adequate, such as in dry conditions, and at an inappropriate temperature or pH. The flow of PB kept the enzyme membrane wet, supplied NADH, and also efficiently removed the reaction products (including NAD^+) and excessive substrates. Hence, reaction conditions were optimized by the circulating PB.

Characterization of the acetone bio-sniffer was carried out by supplying the standard gas to the sensing region via a Teflon tube. High purity air flowed into the Teflon tube first, and subsequently the acetone vapor was applied to the bio-sniffer. The total flow rate was kept at 200 ml min^{-1} . High purity air was also used as the carrier gas to adjust the acetone levels. Changes in NADH fluorescence intensity were measured using a PMT. The experiment was repeated for five cycles.

2.4 The effect of pH and NADH concentration, and bio-sniffer selectivity

212 The bio-sniffer's response to PB of different pH was evaluated first. 50 μ M
213 NADH was added to 0.1 M PB solutions of different pH (pH 5.5, 6.0, 6.5, 6.7, 7.0,
214 7.2 and 7.5) to prepare circulating PB solution. 1 ppm acetone vapor was
215 applied to the bio-sniffer and the prepared solutions of different pH values were
216 circulated into the flow-cell.

217 Subsequently, the effect of NADH concentration was evaluated under
218 optimized pH conditions. Various NADH concentrations (20, 50 and 100 μ M)
219 were prepared in 0.1 M pH 7.0 PB, . 1 ppm acetone vapor was applied to the
220 bio-sniffer and the prepared solutions of different NADH concentration were
221 circulated into the flow-cell.

222 The bio-sniffer's selectivity was then evaluated with eleven single gases
223 and two mixed gases. The following single gases were used at a concentration
224 of 1 ppm: acetone, 2-butanone, 2-pentanone, acetaldehyde, formaldehyde,
225 methanol, 2-propanol, 1-propanol, ethanol, 1-butanol, methyl mercaptan. The
226 mixed gases included 1 ppm acetone and either 0.5 ppb 2-pentanone or
227 2-butanone. These gases are typically found in human breath. Each gas was

supplied from the standard gas generator.

2.5 Measurement of breath acetone during an exercise stress test

Authorization for this part of the study was granted by the Human Investigations Committee of the Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University (authorization code: 2014-01), conforming to standards stipulated in the Declaration of Helsinki. Informed consent was obtained from all subjects.

Subjects attended the laboratory in the morning after an overnight fast of at least 6 hours. Expired air samples were collected at rest at 0 minutes and later at 30 minutes. The subjects then cycled for 30 minutes on a bicycle ergometer, with a constant load of 50 watts. Heart rate was continuously recorded during exercise and expired air samples were collected at 10 minute intervals. Air samples were also collected at 15 or 30 minute intervals after 150 minutes of exercising. Each subject was asked to inspire normally, hold their breath for 5 seconds, and then exhale slowly. Exhalation from the first 3 seconds was

244 discarded and the remainder of expired air was collected using a sampling bag
245 with a mouthpiece (Bikov et al. 2013) The bio-sniffer was subsequently exposed
246 to the samples of expired air using an air pump at a flow rate of 200 ml min⁻¹
247 (S1).

248

249 3. Results and discussion

250 3.1 Characteristics of the acetone bio-sniffer

251 The fluorescent response to acetone vapor was investigated using the
252 standard gas measurement system. Figure 2 shows real-time changes in
253 fluorescence intensity (Δ intensity) at various concentrations of gaseous acetone.
254 When the sensing region was exposed to acetone vapor, fluorescence intensity
255 decreased and reached steady state values immediately. Fluorescence intensity
256 returned to the initial state upon cessation of acetone vapor flow. These
257 responses indicate that NADH decreases at the enzyme membrane as it is
258 consumed through the catalytic reaction involving S-ADH and acetone vapor,
259 and immediately removed from the sensing region by the buffer flow.

The bio-sniffer measures the gas level as a balance between consumption and supply rates of NADH. The optimal flow rate of PB was found to be 1.5 ml min⁻¹. When the PB flow rate is below this value, supplied NADH is insufficient resulting in a smaller Δ intensity at the steady state and a longer recovery time. Conversely, when the PB flow rate is high, supplied acetone vapor is insufficient to fully react with NADH, resulting in a smaller Δ intensity at the steady state and a shorter recovery time. The fast response to acetone vapor confers an advantage in on-site measurement compared to conventional chromatographic methods such as GC and other biochemical alternatives, such as colorimetric methods (Worrall et al. 2013). The time to reach 95% of the steady state was 35–70 seconds; this is on par with solid state gas sensors used for monitoring purposes (Righettoni et al. 2012). Hence, this demonstrates that the bio-sniffer enables real-time monitoring of acetone vapor concentration.

The calibration curve for acetone vapor is shown in Figure 3. The analytical curve follows the sigmoidal shape described by the following equation:

276
$$\Delta \text{intensity (counts)} = 165197 / (1 + (466 / \text{acetone vapor [ppb]})^{0.97})^{0.86} \quad (2)$$

277

278 This equation has a correlation coefficient of 0.999. The dynamic range was

279 from 20 to 5300 ppb, which includes breath acetone concentrations of healthy

280 (200–900 ppb) and diabetic (> 900 ppb) individuals. Fluorescent output of the

281 bio-sniffer also demonstrated high reproducibility. For acetone vapor at 500 ppb,

282 a coefficient of variation of 2.6% (n = 5) was obtained. These values provide

283 evidence supporting the use of the bio-sniffer to measure acetone in exhaled

284 human breath.

285

286 **3.2 The effect of pH and NADH concentration, and bio-sniffer selectivity**

287 To optimize measurement conditions for the acetone bio-sniffer, signal

288 dependence on the pH value of PB and the NADH concentration were

289 investigated. For pH value, $\Delta \text{intensity}$ at different PB pH values were measured

290 for a given acetone vapor concentration. PB pH values were varied from 5.5 to

291 8.0 whilst keeping concentrations of PB and NADH at 0.1 M and 50 μM

respectively. As shown in S2, Δ intensity peaks at pH 7.0 for three different acetone concentrations (250, 500 and 900 ppb). This result can be explained by the following two reasons: Firstly, the enzymatic activity of S-ADH is dependent on PB pH, as pH can alter the three-dimensional conformation of an enzyme and its active site, which influences its stability and efficacy. Hence, pH optimization confers greater stability for the sensor response. Secondly, NADH is an unstable reagent in mildly acidic conditions; hence pH 7.0 is more suitable for NADH stability.

Subsequently, with a PB pH of 7.0, the effect of NADH concentration on the dynamic range for the acetone bio-sniffer was examined. As indicated in Figure 4, for 50 μ M NADH the dynamic range for acetone vapor was from 20 to 5300 ppb. This implies that a low concentration of NADH results in a low background, which is important for measuring acetone at a low concentration. With 20 μ M NADH the dynamic range was lower from 7 to 900 ppb. However, when the concentration of NADH is below 20 μ M there is insufficient NADH for the enzymatic reaction, and sensitivity becomes poorer. On the other hand,

308 when the concentration of NADH is increased to 100 μ M the dynamic range
309 broadens to 50 to 10000 ppb, with a substantial increase at the upper limit. For
310 NADH concentrations exceeding 100 μ M, the upper limit is saturated and the
311 lower limit detrimentally increases. By fine-tuning the NADH concentration, the
312 acetone bio-sniffer can be adjusted to measure acetone vapor at concentrations
313 from 7 to 10000 ppb.

314 The selectivity of the acetone bio-sniffer was also determined. Since
315 NADH-dependent S-ADH is reactive to ketones, 2-butanone, 2-pentanone and
316 other aldehydes, thirteen different gaseous species including eleven single
317 gases and two mixed gases were tested to compare responses. Figure 5 shows
318 relative changes in fluorescence intensity for various gaseous species in
319 comparison to acetone vapor. Fluorescence intensities for 2-butanone and
320 2-pentanone exceeded that of acetone, reaching 139% and 117% respectively,
321 while little fluorescence was detected for other single gases. This demonstrates
322 that the bio-sniffer exhibits high selectivity due to substrate specificity of the
323 enzyme.

Although high responses to 2-butanone and 2-pentanone were observed, the output interference is negligible as only minute quantities of these gases (0.38 ppb) are present in the exhaled breath of healthy people (Van den Velde et al. 2008). Evidence also indicates that 2-butanone and 2-pentanone are volatile biomarkers of lung cancer found in the exhaled breath of lung cancer patients (Buszewski et al. 2012). Given the high responses to both gases, the acetone bio-sniffer might have the potential to be used as a diagnostic tool for lung cancer screening.

3.3 Breath acetone analysis during the exercise stress test

Unlike chromatographic techniques, the acetone bio-sniffer enables real-time measurement of acetone vapor. We harnessed this function to assess lipid metabolism based on breath acetone analysis. Figure 6 depicts the time courses of acetone concentration in exhaled breath before, during and after the exercise stress test. Subjects were fasted for at least 6 hours beforehand. Acetone concentrations increased during exercise, and peaked at 15–30

minutes after exercise. At the peaks, acetone concentrations reached 110–140% of the initial values. Thereafter, they gradually decreased to steady states.

Elevated acetone concentration in exhaled breath correlates with increased lipolysis due to the exercise. During exercise after a period of fasting, fatty acids are released from adipose tissue and enter the bloodstream as free fatty acids (FFAs). Increased FFAs are broken down by beta-oxidation to produce acetyl CoA, from which ketone bodies (acetoacetate, beta-hydroxybutyric acid and acetone) are synthesized. Volatile acetone can then enter alveolar airspaces during gaseous exchange, and is consequently exhaled. After exercise, ketone body concentration in blood continues to increase. Thus, breath acetone concentration peaked after the exercise, and only decreased thereafter. This proof of concept demonstrates that the acetone bio-sniffer can be used to assess lipid metabolism during exercise.

4. Conclusion

In this study, we developed a highly sensitive and selective biosensor for

the measurement of acetone in exhaled breath based on the reverse (reduction) catalytic reaction of S-ADH. The acetone bio-sniffer employed a UV-LED as a light source to facilitate miniaturization and ease of use. Real-time monitoring of acetone vapor was successfully performed using a cylindrical flow-cell with an enzyme immobilized membrane. The fluorescence signal exhibited a clear relationship with acetone concentration from 20 to 5300 ppb; this encompasses acetone concentrations in the exhaled breath of both healthy and diabetic individuals. The response time to reach 95% of the steady state was 35–70 seconds. Furthermore, the dynamic range of the bio-sniffer could range from 7–10000 ppb by fine-tuning the NADH concentration. Since the bio-sniffer also detects 2-butanone and 2-pentanone, it could potentially be used as a diagnostic tool for lung cancer screening. When assessing lipid metabolism based on exhaled breath, increased acetone concentration due to exercise was observed. We anticipate future use of the acetone bio-sniffer as a non-invasive analytical tool for assessment of lipid metabolism in exercise, fasting and diabetes mellitus.

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376 Education, Culture, Sports, Science and Technology (MEXT)

377

378 **Figure captions**

379 **Figure 1**

380 Experimental setup for characterization of the fiber-optic acetone bio-sniffer. The
381 bio-sniffer measures acetone vapor as fluorescence of NADH, which is
382 consumed at the enzyme membrane. PB that contained NADH was circulated at
383 a flow-rate of 1.5 ml min^{-1} . The capacity of the reservoir is 1000 ml. The inset
384 shows the cross-sectional structure of the flow-cell with the S-ADH immobilized
385 membrane.

386

387 **Figure 2**

388 Changes in various concentrations of standard acetone vapor over time. The
389 steady state value is determined by the balance consumption rates of NADH.
390 The response time to reach 95% of the steady state was 35–70 seconds.

391

392 **Figure 3**

393 Calibration curve for the acetone bio-sniffer using acetone vapor. The
394 fluorescence intensity relates to the concentration of acetone vapor from 20 to

395 5300 ppb.

396

397 **Figure 4**

398 Effect of the concentration of NADH on the range of the acetone bio-sniffer. For

399 50 μ M NADH the dynamic range for acetone vapor was 20 to 5300 ppb. For 20

400 μ M NADH the dynamic range was 7 to 900 ppb. Increasing the concentration of

401 NADH to 100 μ M improves the upper limit of the dynamic range and expands it

402 to 50 to 10000 ppb.

403

404 **Figure 5**

405 Gas selectivity of the S-ADH bio-sniffer. The bio-sniffer response to 2-butanone

406 and 2-pentanone was 139% and 117% respectively. A smaller response was

407 detected for other chemical substances.

408

409 **Figure 6**

410 Time course of acetone concentration in exhaled breath before, during and after

411 exercise. The subjects were fasted for at least 6 hours before exercising. Breath
412 acetone concentrations increased during exercise, and peaked 15–30 minutes
413 after exercise. At the peaks, acetone concentrations reached 110–140% of the
414 initial values.

415

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Figures:

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6