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Determination of anabolic steroids with gas chromatography–ion trap mass spectrometry using hydrogen as carrier gas

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Helium is considered to be the ideal carrier gas for gas chromatography/mass spectrometry (GC/MS) in general, and for use with an ion trap in particular. Helium is an inert gas, can be used without special precautions for security and, moreover, it is needed as a damping gas in the trap. A disadvantage of helium is the high viscosity resulting in long GC run times. In this work hydrogen was tested as an alternative carrier gas for GC in performing GC/MS analyses. A hydrogen generator was used as a safe source of hydrogen gas. It is demonstrated that hydrogen can be used as a carrier gas for the gas chromatograph in combination with helium as make-up gas for the trap. The analysis time was thus shortened and the chromatographic performance was optimized. Although hydrogen has proven useful as a carrier gas in gas chromatography coupled to standard detectors such as ECD or FID, its use is not mentioned extensively in the literature concerning gas chromatography–ion trap mass spectrometry. However, it is worth considering as a possibility because of its chromatographic advantages and its advantageous price when using a hydrogen generator.

For many years, hydrogen (mentioned in the tables and figures as H_2) has been commonly used as a carrier gas in gas chromatography coupled to ECD,[1] FID[4–7] or other detection techniques.[2,8] It offers important advantages over helium in terms of speed of analysis, sensitivity and resolution.[9,10] It is, however, potentially explosive due to characteristics that make it hazardous to apply: at atmospheric pressure hydrogen is combustible at concentrations in air from 4% to 74.2% by volume, it has the highest burning velocity of any gas, it has very low ignition energy and it can self-ignite when expanding rapidly from high pressure. Placing a hydrogen gas cylinder near instruments in a laboratory is excluded for security reasons. Possible solutions are hydrogen lines or a hydrogen generator.[1] The first solution can be rejected because of the potential danger for leaks.

The hydrogen generator produces hydrogen (and oxygen as by-product) through the electrolysis of deionized, distilled water. The key element of the generator is an electrochemical cell assembly that contains a solid polymer electrolyte. There are no free acids or alkalis used. Demineralized or distilled water is the only liquid contained in the unit. The hydrogen generator tested in this investigation can produce up to 160 mL/min H_2 (measured at 20 °C and 1.013 bar) and is thus able to serve two or even three analytical systems. Purities up to 99.9999% are available and the generator offers silent operation at pressures up to 7 bar. Commonly, hydrogen gas is produced at a pressure of 3 bar.

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Helium (mentioned in the tables and figures as He) is most generally considered to be the ideal carrier gas for gas chromatography/mass spectrometry (GC/MS). It is an inert gas and can be used without special precautions for security. In ion trap mass spectrometry helium is needed as a damping gas in the ion trap. A disadvantage of helium is its higher viscosity resulting in long GC run times. Moreover, helium gas at the purity needed for GC/MS is expensive and, due to a higher demand than supply, the prices are still rising. In view of the fact that in splitless analyses using a split/splitless injector the split flow is constant (flow rate 60 mL/min), and the splitting line is only closed during sample transfer onto the column, a lot of expensive helium is blown into the air.

To test whether H_2 gas can be used as an alternative carrier gas for GC/MS and GC/MS^2, a hydrogen generator was coupled to a gas chromatograph combined with an ion trap. Standards of anabolic steroids were analyzed in electron impact full scan mode (GC/EI-MS) and in full scan GC/EI-MS^2 mode.

EXPERIMENTAL

Reagents and chemicals

The following reagents and solvents were of analytical grade quality and provided by Merck (Darmstadt, Germany): ethyl acetate, methanol, and absolute ethanol. Perfluorotributylamine (FC-43) (Ultra Scientific, North Kingstown, USA) was used as MS calibrant. The derivatization reagent ($MSTFA^{++}$) was prepared by dissolving 100 mg ammonium iodide (NH_4I) (Sigma, St. Louis, MO, USA) and 0.2 mL ethanethiol

RCM

Table 1. Inlet pressure of the carrier gas for GC is dependent on the flow rate of the carrier gas used

Flow rate	Inlet pressure He	Inlet pressure H_2
1 mL/min	1.0630 bar	0.3154 bar
2 mL/min	1.8435 bar	0.8665 bar
3 mL/min	2.4853 bar	1.2896 bar

(Acros, Geel, Belgium) in 5 mL *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) (Macherey-Nagel, Düren, Germany), followed by dilution of 1.5 mL of this solution with 10 mL MSTFA.

Most steroids were obtained from Steraloids (Wilton, NY, USA) or Sigma (St. Louis, MO, USA). Other steroids were gifts from various sources. All recent standards were obtained through the National Reference Laboratory, WIV-LP, Brussels, Belgium, and were developed to ensure that all the field laboratories use the same standards.[11]

Gas chromatography (GC) and coupled mass spectrometry (GC/MS)

The ion trap used was a POLARIS mass spectrometer coupled to a Trace GC gas chromatograph, both from ThermoFinnigan (Austin, USA). Samples were injected using an autosampler AS2000 (ThermoFinnigan). A hydrogen generator (Packard, Meriden, USA) was coupled to the gas chromatograph and hydrogen gas was used as carrier gas for GC; the inlet pressure was dependent on the flow rate of the carrier gas used (Table 1). The analyses were performed using a non-polar 5% phenyl polysilphenylene-siloxane SGE BPX-5 column (25 m × 0.22 mm i.d. 0.25 μm), with constant flow and temperature programming from 100 °C (held for 1 min) to 250 °C at 17 °C/min and to 300 °C at 2 °C/min. A split/splitless injector (split flow 60 mL/min) was set at 250 °C and the transfer line at 275 °C. The same experiment was performed for comparison using helium as the carrier gas for GC.

In both experiments MS/MS measurements were performed using helium as collision gas in the ion trap at a supply pressure of 3 bar with an ionizing electron energy of 70 eV. Xcalibur® software (ThermoFinnigan, Austin, USA) version 1.2 was used to perform the data processing and interpretation of the analytical results.

Standard solutions

From the stock solutions (200 ng anabolic steroid/μL absolute ethanol, stored at 4 °C) a solution containing several analytes was prepared. The concentration of each component in this mixture solution depended on its limit of detection. The anabolic steroids were derivatized to enol trimethylsilyl ether derivatives with $MSTFA^{++}$: 10 μL of the standard mixture solution were transferred into an autosampler vial and evaporated to dryness under nitrogen flow (e.g. by using a Turbovap). Then $MSTFA^{++}$ (25 μL) was added, the contents were mixed and incubated for 1 h at 62 °C and a 1μL aliquot of the derivatized solution was injected into the GC column.

Analytical experiments

After the acquisition of a full scan (GC/EI-MS) analysis using helium as carrier gas for GC, a hydrogen generator was coupled to the gas chromatograph and the steroid standards were analyzed in the same way with H_2 at flow rates of 1, 2 and 3 mL/min. The dependence on flow rate of

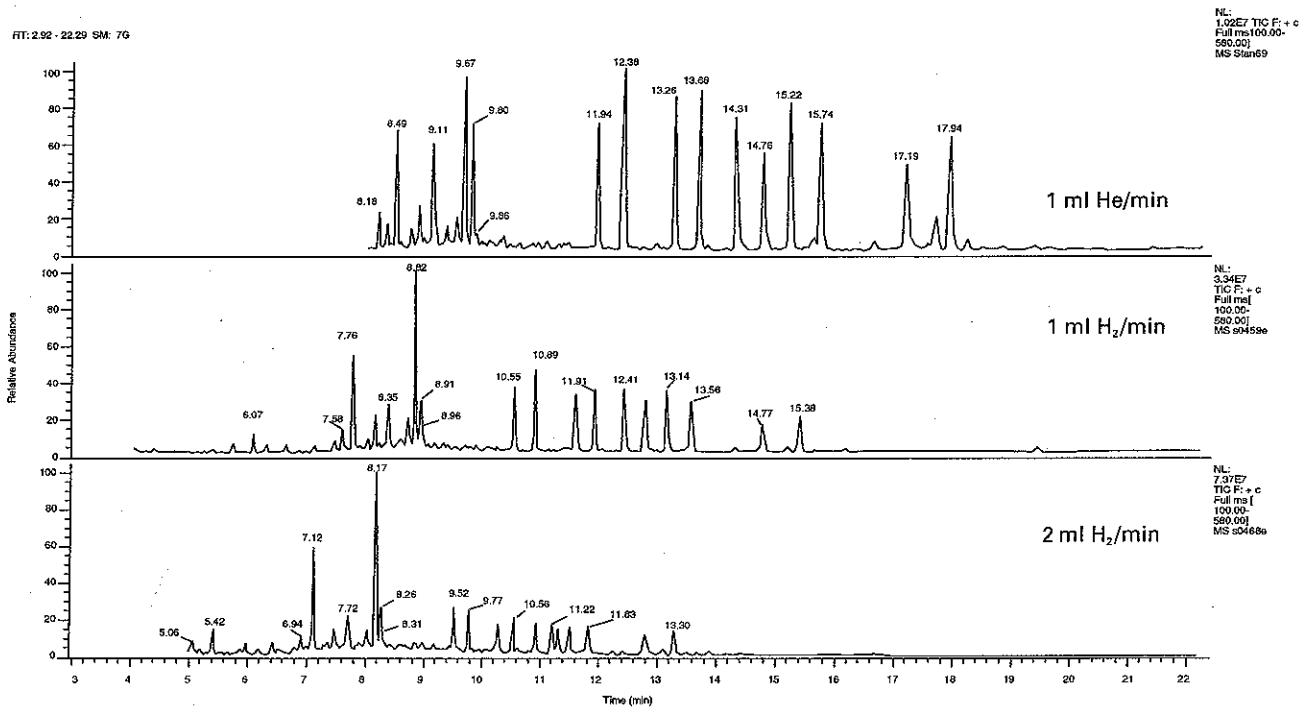


Figure 1. Comparison of GC run times for a standard solution of steroids, using (a) He at 1 mL/min, (b) H_2 at 1 mL/min, and (c) H_2 at 2 mL/min. All other parameters were fixed.

Table 2. Comparison of the absolute retention times (Rt) of some anabolic steroids obtained using full scan GC/MS with He (flow rate 1 mL/min) and H_2 (flow rate 1 mL/min (a) and 2 mL/min (b))

Component	Rt He	Rt H_2 (a)	Rt H_2 (b)
Androsterone	12.38	10.89	9.77
alfa-Nortestosterone	13.26	11.58	10.2
alfa-Testosterone	13.68	11.91	10.56
beta-Testosterone	14.31	12.41	10.90
Methandriol	14.77	12.77	11.22
Methylnortestosterone	15.21	13.14	11.50
Acetoxyprogesterone	15.22	13.16	11.52
Methyltestosterone	15.74	13.56	11.83
Norgestrel	17.19	14.77	12.88
Chloroandrostenedione	17.94	15.38	13.30

the absolute retention time of each analyte was determined. Also the chromatographic resolution was evaluated.

Some anabolic steroids (e.g. norgestrel) were also analyzed by full scan GC/EI-MS^2 using hydrogen as carrier gas at a flow rate of 1 mL/min. Component-specific precursor ions were fragmented with component-specific voltage energy. The results were compared with those of an equivalent analysis using helium at the same flow rate. In all experiments helium was needed as damping gas (and supplied directly) into the ion trap.

RESULTS AND DISCUSSION

Generally anabolic steroids are analyzed in full scan mode (GC/EI-MS) using helium as carrier gas at a flow rate of 1 mL/min. Using hydrogen as an alternative carrier gas, GC/EI-MS can be performed at flow rates of 1, 2 and 3 mL/min. In performing analyses using a gas chromatograph coupled to an ion trap, the first two flow rates are useful, but at 3 mL/min the pressure in the mass spectrometer becomes quite high. At flow rates higher than 3 mL/min the mass spectrometer fails as the pressure in the mass analyzer region is too high to ensure good enough vacuum conditions, resulting in a shut down of the instrument.

Figure 1 compares the chromatograms obtained using full scan MS and helium (flow rate 1 mL/min) and hydrogen (flow rates 1 and 2 mL/min) as carrier gas. Here, the total ion current is a determining parameter for interpretation. As predicted, the run time for analysis of the steroids in the mixture can be shortened using hydrogen, and the higher the flow rate the shorter the analysis time. However, the flow rate should not be too high since, due to the decrease in the analysis time, all analytes elute at earlier retention times, resulting in a more condensed chromatogram. When the number of analytes is too high, the chromatographic resolution is not satisfactory at a high flow rate. A flow rate of 1 mL/min was considered ideal to obtain optimal chromatograms. In Table 2, a comparison of the absolute retention times of some anabolic steroids obtained using full scan GC/MS with helium (flow rate 1 mL/min) and with hydrogen (flow rates 1 and 2 mL/min) is made: a decrease in retention times by approximately 25% can be established if working at a flow rate of 2 mL/min.

In Fig. 2 the chromatographic resolution is evaluated. In a chromatogram, resolution between two adjacent peaks is defined as the distance between the centers of the peaks divided by their average peak width (measured at 10% of the peak height). A resolution (Rs) of 1.00 indicates baseline separation. Application of this definition of resolution to the chromatograms shown in Fig. 2, for which the component-specific ions (*m/z* 307, 335, 389 and 433) of α-Zeranol and β-Zeranol are the determining parameters for interpretation of a full scan GC/MS analysis, resulted in a Rs value of 1.59 for analyses using helium, and a Rs value of 2.09 using

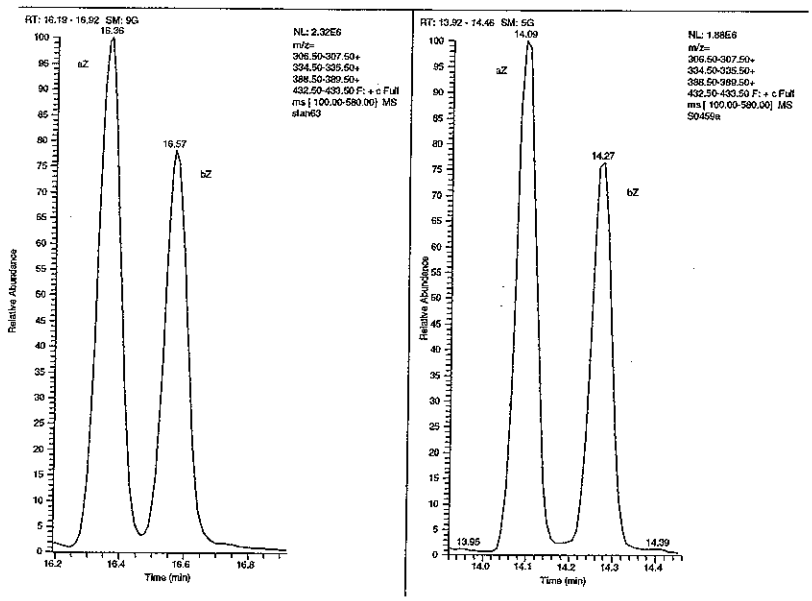
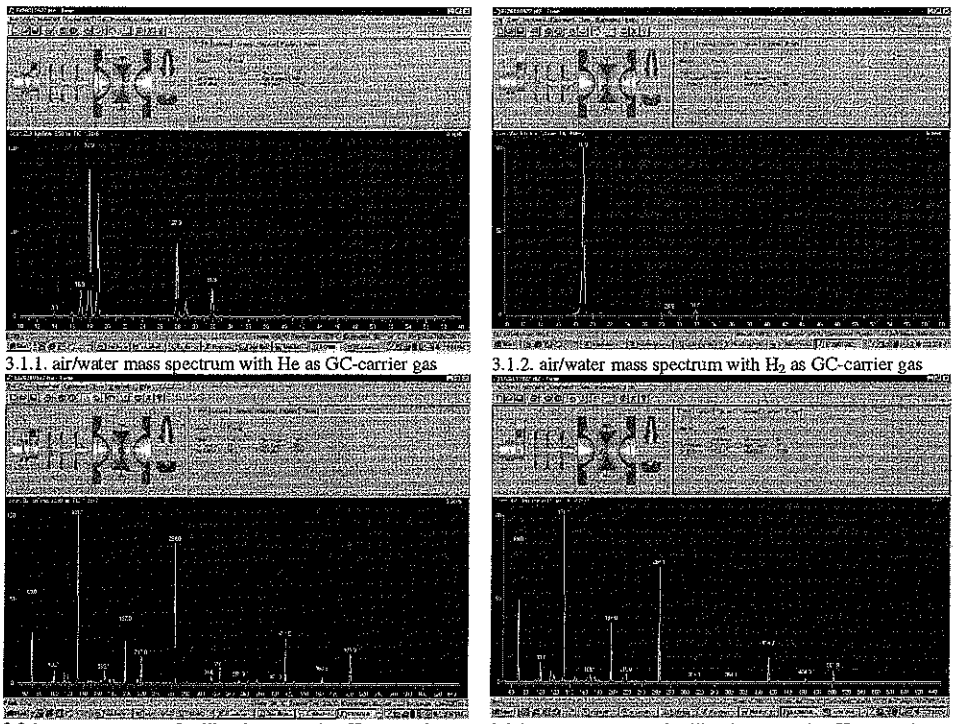


Figure 2. Comparison of chromatographic resolution using helium (left chromatogram) and hydrogen (right chromatogram) for α-Zeranol (at retention time 16.36 min, resp. 14.09 min) and β-Zeranol (at retention time 16.57 min, resp. 14.27 min).

RCM



3.1.1. air/water mass spectrum with He as GC-carrier gas

3.1.2. air/water mass spectrum with H_2 as GC-carrier gas

3.2.1. mass spectrum of calibration gas using He as carrier gas

3.2.2. mass spectrum of calibration gas using H_2 as carrier gas

Figure 3. Comparison of tune mass spectra using helium (3.1.x) and hydrogen (3.2.x) with identical tune parameters for both GC carrier gases.

hydrogen as carrier gas, i.e., the chromatographic resolution for hydrogen is better than that obtained when working with helium. Further interpretation of both chromatograms confirms the findings of Tunesi-Claind[10] in 1998, i.e., hydrogen as carrier gas for capillary gas chromatography offers important advantages over helium in terms of efficiency, resolution and speed of analysis.

Some attention must be paid to the MS conditions when using hydrogen as carrier gas for a gas chromatograph coupled to an ion trap mass spectrometer. In determining

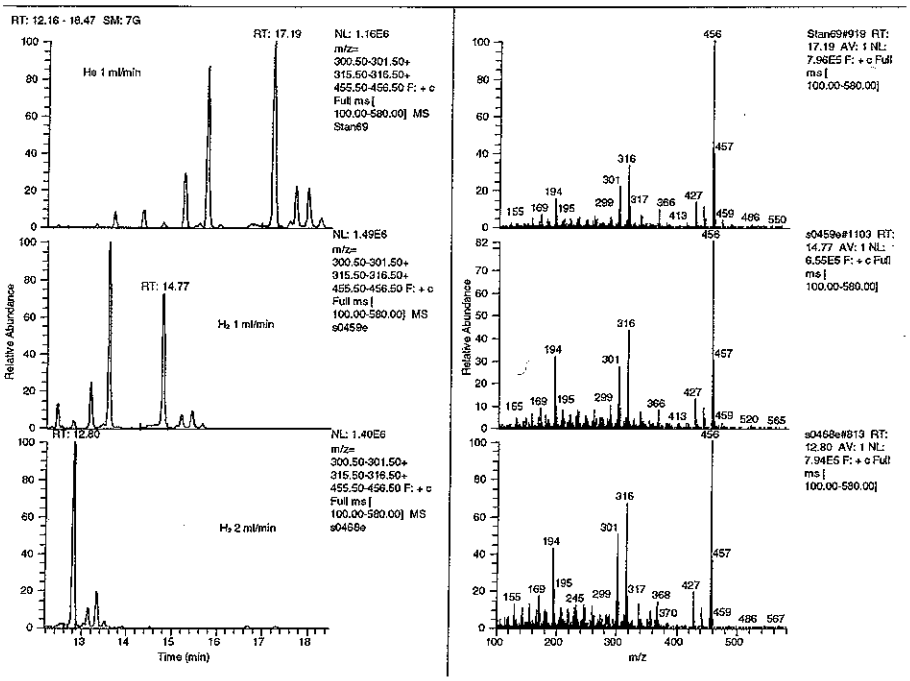


Figure 4. Full scan GC/MS analysis using helium (flow rate 1 mL/min) and hydrogen (flow rates 1 mL/min and 2 mL/min) as carrier gas.

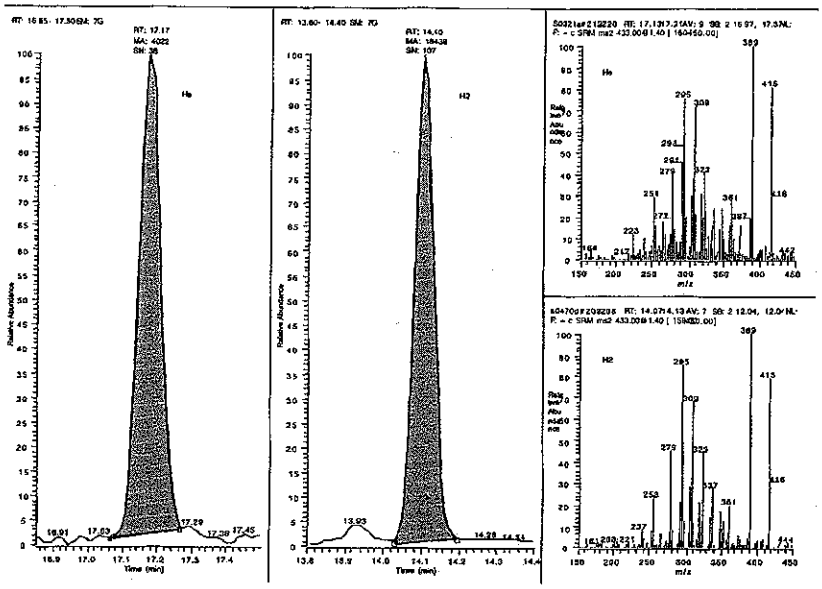


Figure 5. Comparison of typical chromatograms and mass spectra of β-Zeranol (0.1 ng/μL) using He (left chromatogram, top spectrum) and H_2 (right chromatogram, bottom spectrum) as carrier gases in full scan GC/MS^2 mode.

optimal MS conditions the air/water leak rate in the ion trap is a decisive factor, because these two contaminants must be reduced as much as possible to obtain reliable analytical results. In Fig. 3, the impact of hydrogen (used only in GC) on MS is illustrated. For both carrier gases all the MS parameters were identical. On the left side (Fig. 3.1.1) the air/water spectrum using helium shows an ideal situation: two peaks at *m/z* 17.9 and 18.9 refer to water (H_2O), and peaks at *m/z* 27.9 and 31.9 refer to the nitrogen (N_2) and oxygen (O_2) portion of air, with a $H_2O/N_2/O_2$ in a ratio (9:6:3) corresponding to optimal MS conditions. The peaks are narrow and well resolved. When using hydrogen the air/water spectrum looks different because there is just one water peak at *m/z* 18.9 and the $H_2O/N_2/O_2$ ratio is much higher (Fig. 3.1.2). Since hydrogen is introduced into the mass spectrometer at a flow rate of 1 mL/min, and helium is needed as damping gas in the ion trap, the higher the flow rate of entering hydrogen the higher the concentration of hydrogen will be in the ion trap, and thus the air/water spectrum will become far from optimal for MS conducive to performing reliable GC/MS analyses. Also, when the water concentration in the mass spectrometer becomes too high, the system will automatically shut down to prevent the filament from burning through. As mentioned earlier, a hydrogen flow rate of 1 mL/min in the gas chromatograph (and thus in the mass spectrometer) corresponds to the generally optimal conditions in practice. In Figs 3.2.1 and 3.2.2 the mass spectra for the MS calibration gas (perfluorotributylamine) are shown. The fragmentation patterns are similar whether helium or hydrogen is used as carrier gas.

In Fig. 4 the results for norgestrel are presented. Norgestrel is one of the gestagenic steroids considered as 'difficult' for full scan GC/MS analysis. Here interpretation was performed by selection of the norgestrel-specific fragment ions (*m/z* 456, 316 and 301) in the full scan mass chromatogram. Examination of the mass spectra shows that the relative fragment ion intensities are higher when using hydrogen as carrier gas. The combination of hydrogen and helium in the ion trap results in a higher degree of fragmentation, and the ion trap spectra resemble more those obtained using a quadrupole mass analyzer.

The investigation also investigated the possibility of using H_2 as carrier gas in performing analyses in full scan MS^2 mode. Figure 5 shows typical chromatograms and mass spectra of β-Zeranol (0.1 ng/μL; 100 pg injected), using helium and hydrogen as carrier gas. The isolated precursor ion at *m/z* 433 was fragmented with 1.40 V activating potential (details in Table 3). A 'cleaner' fragment ion spectrum, with somewhat more component-specific fragmentation, was achieved using hydrogen. The other steroids tested in this investigation indicated the same trend.

Although hydrogen seems to be an ideal gas for GC/MS when using an ion trap, some disadvantages should be mentioned. An additional hydrogen sensor in the GC oven (with additional costs) is required, and tuning of the mass spectrometer is more difficult and time-consuming. Concerning the ion trap mass spectrometer, the ion source and mass analyzer seem to be contaminated earlier if using hydrogen as carrier gas instead of helium, but there is no certainty that the GC-hydrogen is solely responsible.

Table 3. Parameters used to perform GC/MS^2 analysis of β-Zeranol

Parameter	Value
Isolation precursor ion (*m/z*)	433
Isolation width (u)	1.0
Isolation time (ms)	8.0
Excitation voltage energy (V)	1.40
Excitation time (ms)	15.0
Maximum excitation energy (q)	0.30
Product ions (*m/z*)	155–433

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CONCLUSIONS

The work presented above has demonstrated the usefulness of hydrogen as a carrier gas for GC/MS or GC/MS^2 analyses with an ion trap. With a hydrogen generator and detector on the gas chromatograph most of the disadvantages of hydrogen are minimized. Instead of the need to buy expensive helium or dangerous hydrogen gas cylinders, the generator is filled with essentially free ultra-pure water. Because the run time of one analysis can be shortened, more samples can be analyzed, resulting in a faster report of the results. Moreover, in general, more abundant fragmentation is observed, and also higher relative abundances of the diagnostic ions for identification of the components.

This experimental arrangement using hydrogen as an alternative carrier gas has been in use for routine analysis in our laboratory for six months now. With this arrangement, the detection limits demanded by government regulations for most estrogens, androgens and gestagens of the Belgium national plan have been validated.

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