The influence of temperature on the separation and characterization of steroids on non-polar glass capillary columns

J. KRUPČÍK*, G. A. F. M. RUTTEN, and J. A. RIJKS

Department of Instrumental Analysis, University of Technology, Eindhoven, The Netherlands

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A precise gas chromatograph for analysis of high boiling compounds such as steroids is described. Standard deviation of Kováts indices of some steroid hydrocarbons, TMS and MO—TMS derivatives of steroids on non-polar coated glass capillary columns is less than ± 0.5 index unit. This repeatability permits to study the temperature dependence of Kováts indices of steroids. It is demonstrated that some overlapped peaks can be resolved by changing the column temperature.

It is shown that $\partial I/\partial T$ values of TMS and MO—TMS derivatives of steroids depend on steric configurations, nature of functional groups, and on their positions in skelet. $\partial I/\partial T$ values could be valuable for the characterization of steric isomers, the mass spectra of which are not sufficient enough for identification purposes.

Приводится описание прецизионного газового хроматографа для анализа высококипящих соединений на подобие стероидов. Стандартные отклонения показателей Коватса для некоторых стероидных углеводородов, TMS и MO—TMS производных стероидов на стеклянных капиллярных колонках с неполярным покрытием составляют меньше $\pm 0{,}5$ единиц показателей. Такая воспроизводимость позволяет изучать температурную зависимость показателей Коватса у стероидов. Демонстрируется способность размещения перекрывающихся пиков с изменением температуры колонки.

Показано, что значения $\partial I/\partial T$ у TMS и MO—TMS производных стероидов зависит от стерической конфигурации, природы функциональных групп и их расположения в системе. Значения $\partial I/\partial T$ могут быть полезной характеристикой тех стерических изомеров, чьи масс-спектры недостаточно подходят для целей идентификации.

Gas chromatography is one of the most widely used and effective techniques for the analysis of hormonal steroids in biological samples. The development in this field has mainly resulted from recent advance made in the preparation of open tubular glass capillary columns [1—7] and the coupling of gas chromatography and mass spectrometry [8, 9]. In many cases this combination can be successfully

* *Present address:* Department of Analytical Chemistry, Slovak Technical University, 880 37 Bratislava, Czechoslovakia.

applied for the identification of steroids in natural samples. Although for positional and sterical isomers, giving rise to similar mass fragmentation patterns, it is desirable to have means available for the characterization by retention data only. Therefore the separation power of the column and the accuracy of the retention data have to be improved. In order to extend this possibility of steroid identification, which is also important in laboratories where a GC—MS combination is not available, the influence of improvement of instrumentation and the measurement of retention data is studied. Additional information that can be obtained from differences in temperature dependence of retention of some steroids will be discussed.

Experimental

Instruments

In this study the following gas chromatographs were used:

CH1: A self assembled gas chromatograph with a flame ionization detector and a direct all glass solid injection system [10] mounted at a "syndanio" plate, placed on the upper part of an air thermostat (Model 1452 DPT, Becker Delft, The Netherlands). The column was connected to the injection system and to a glass adapter at the detector side by shrinkable teflon tubings. The adapter at the detector side tapered off to a capillary penetrating into the top of the flame (minimum dead volume). The pressure drop across the column was controlled by 2 pressure regulators (Model MB 19936, Becker Delft, The Netherlands) placed in series. The variation of the pressure drop, during one analysis, was better than 0.2 kPa.

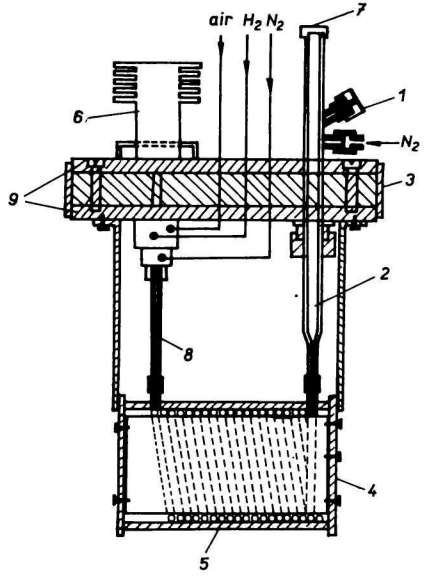

Fig. 1. Scheme of the modified upper part of Becker 1452 DPT thermostat.
1. Introduction of samples; *2.* all-glass solid injection system; *3.* metal ring; *4.* aluminium block; *5.* glass capillary column; *6.* FID; *7.* restriction capillary; *8.* glass capillary; *9.* "syndanio" double lid.

CH2: A self assembled gas chromatograph similar to *CH1*. To decrease the temperature gradient in the upper part of the thermostat the cover was composed of two "syndanio" plates with a heating element between them. The injection system and flame ionization detector penetrated into the thermostat *via* this double lid. The glass capillary column was placed inside an aluminium block (Fig. 1), consisting of a massive aluminium cylinder covered by an aluminium mantle. To minimize the dead volume, nitrogen was added as a purge gas before the detector was joined.

In this way a homogeneous column temperature could be obtained within 0.2 K at 503 K, during each analysis and 0.4 K in 24 hrs.

Glass capillary columns

From different available materials for column preparation, glass seems to be the only one of interest for the separation of steroids. In spite of the low activity of glass, it still needs a pretreatment prior to the coating of the column with non-polar stationary phases. *Rutten* and *Luyten* [5] as well as *German* and *Horning* [7] published recently successful preparation of glass capillary columns with non-polar polysiloxane phases (SE-30 and OV-101).

Preparation of glass capillary columns

Capillary columns were drawn from pyrex glass tubes. Empty columns were rinsed with CH_2Cl_2, deactivated with a solution of BTPPC (benzyltriphenyl phosphonium chloride) in CH_2Cl_2. The excess of BTPPC was removed by rinsing with CH_2Cl_2. After drying with nitrogen and an intermediate test the columns were coated with solutions of OV-101 in hexane. More details about this method of column preparation are given by *Rutten* and *Luyten* [5].

Column A: Length 30 m, i. d. 0.25 mm. The column was rinsed with CH_2Cl_2 deactivated by a solution of 1% BTPPC in CH_2Cl_2 for 24 hrs. The excess of BTPPC was removed with 2 ml of CH_2Cl_2. Next the column was dried by nitrogen and coated by 0.25% solution of OV-101 in hexane by the static method.

Column B: Length 50 m, i. d. 0.25 mm. The column was prepared using the procedure described before. The stationary phase was OV-101.

Model mixtures

Model mixtures of silylated steroids (TMS derivatives) having a concentration of 10 μg/ml were prepared by reacting the steroids with a mixture of *N,O*-bis(trimethylsilyl)fluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) in a ratio 5:1 (v/v). The methoxime trimethylsilyl derivatives of steroids (MO—TMS derivatives) were prepared by a reaction with methoxy amine hydrochloride in anhydrous pyridine followed by the trimethylsilylation procedure described above [11]. Model mixtures of *n*-alkanes were prepared in concentrations of 10 μg/ml *n*-pentane. These mixtures were injected in amounts corresponding to 2.5—20 ng for each compound. Abbreviations of compounds used in the model mixtures are given in Table 1.

Detection, data read out and data handling

The FID detector was coupled to a low noise home-made amplifier (noise level 0.5×10^{-14} A). The detector signal was registered both with a stripchart recorder and a data acquisition system with subsequent off-line data processing. Reading of the analog signal was taken by a digital voltmeter at a

Table 1

Abbreviations of compounds used in the model mixtures

Trivial name	Systematic name	Abbreviation
	Docosane	C_{22}
	Tetracosane	C_{24}
	Hexacosane	C_{26}
	Octacosane	C_{28}
	5α-Androstane	A_a
	5β-Androstane	A_b
	5α-Pregnane	P_a
	5β-Pregnane	P_b
	5α-Cholestane	CH_a
	5β-Cholestane	CH_b
Androsterone	5α-Androstane-3α-ol-17-one	A
Etiocholanolone	5β-Androstane-3α-ol-17-one	E
Dehydroepiandrosterone	5-Androstene-3β-ol-17-one	DA
Testosterone	4-Androstene-17β-ol-3-one	T
Pregnanolone	5-Pregnane-3α-ol-20-one	Pn
Estradiol	1,3,5(10)-Estratriene-3,17β-diol	E_{II}
11-Hydroxyetiocholanolone	5β-Androstane-3α,11β-diol-17-one	11 OE
Allo-pregnanediol	5α-Pregnane-3α,20α-diol	aPD
Pregnanediol	5β-Pregnane-3α,20α-diol	PD
Estriol	1,3,5(10)-Estratriene-3,16α,17β-triol	E_{III}
Squalene	2,6,10,15,19,23-hexamethyl-2,6,10, 14,18,22-tetracosanhexaene	SQ

preset rate (0.1 or 1 s). When the reading was taken, the digital voltmeter sent a conversion complete signal to a serializer. After serializing, the measurement was punched on a paper tape. The retention time of the peaks was calculated on the basis of a five point fit as described by *Wijtvliet* [12].

Retention times were also partly obtained by using a stop-watch.

Results and discussion

Precision of retention data

Parameters for steroid identification

In gas chromatography of steroids a number of relative retention data can be used for identification purposes, *e.g.* relative retention times, methylene units, steroid numbers and Kováts indices.

For reasons of general use and precision retention data based on more than one standard should be preferred. For precise measurements of retention data in isothermal analysis we have chosen the Kováts retention index. An advantage of

this quantity is the additional information that possibly may enhance the characterization of a compound by using stationary phases of different polarity (ΔI) of one stationary phase at different temperatures ($\partial I/\partial T$) as shown for hydrocarbons [13].

Determination of the "dead time"

Since the introduction of gaseous samples by the all-glass solid injection system is not possible, the "dead time" must be determined indirectly. Calculation of the dead time from three equidistant alkanes according to *Petterson* and *Hirsch* [14] may result into the considerable errors because of slight fluctuation of experimental conditions (extrapolation through more than 20 carbon atoms). Another possibility at higher temperatures is to use the *n*-pentane peak maximum instead of methane. Comparing retention times of methane and pentane at 503 K using an inlet splitter (Hamilton) a difference within the standard deviation of the measurements was found.

Influence of experimental factors

The main experimental factors that affect the precision of retention data are the column in- and outlet pressure, the temperature and the method of "dead time" determination [13, 15].

Table 2

Repeatability of retention data of TMS derivatives of some steroids and normal alkanes measured on instruments *CH1* and *CH2* using column *A* at 503 K

Compound	*CH1*			*CH2*		
	σ_{t_R}	σ_r	σ_I	σ_{t_R}	σ_r	σ_I
C_{22}	0.67	2.3	0	0.03	0.07	0
C_{24}	0.73	1.1	0	0.08	0.05	0
A	0.47	0.4	1.2	0.11	0.04	0.16
E	0.49	0.3	1.2	0.10	0.08	0.04
DHEA	0.57	0.1	0.7	0.11	0.18	0.20
C_{26}	0.54	0	0	0.13	0	0
Pn	0.32	0.2	0.5	0.11	0.12	0.16
E_{11}	0.56	0.3	0.3	0.12	0.15	0.17
11 OE	0.50	0.4	0.3	0.14	0.12	0.23
aPD	0.46	0.6	0.2	0.14	0.12	0.20
PD	0.35	0.8	0.5	0.15	0.10	0.19
C_{28}	0.42	0.7	0	0.15	0.12	0

σ_{t_R} and σ_r — relative standard deviation of the absolute retention time (t_R) and the relative retention time (r) (standard, *n*-hexacosane).

σ_I — absolute standard deviation of Kováts indices.

The repeatability, expressed as the standard deviation, obtained on instrument *CH1* (improved pressure control) and on instrument *CH2* (improved pressure and temperature control) is given in Table 2.

It may be concluded that a repeatability corresponding to the standard deviation of 0.2 index unit can be obtained for a temperature control within 0.1 K and a pressure control better than 0.2 kPa on one column.

For different columns a systematic difference of retention data of about 1 index unit was found. This result clearly shows that the reproducibility of column preparation has to be improved to enable precise measurements of retention data.

The influence of temperature on the separation of steroids

The separation of a model mixture of steroid hydrocarbons and TMS derivatives of steroids at 503 K (stationary phase OV-101) is shown in Fig. 2. The *cis* (5β) isomers of the steroid hydrocarbons elute before the corresponding *trans* (5α) isomers. For the silanized 3α-hydroxy derivatives the elution order of the *cis-trans*

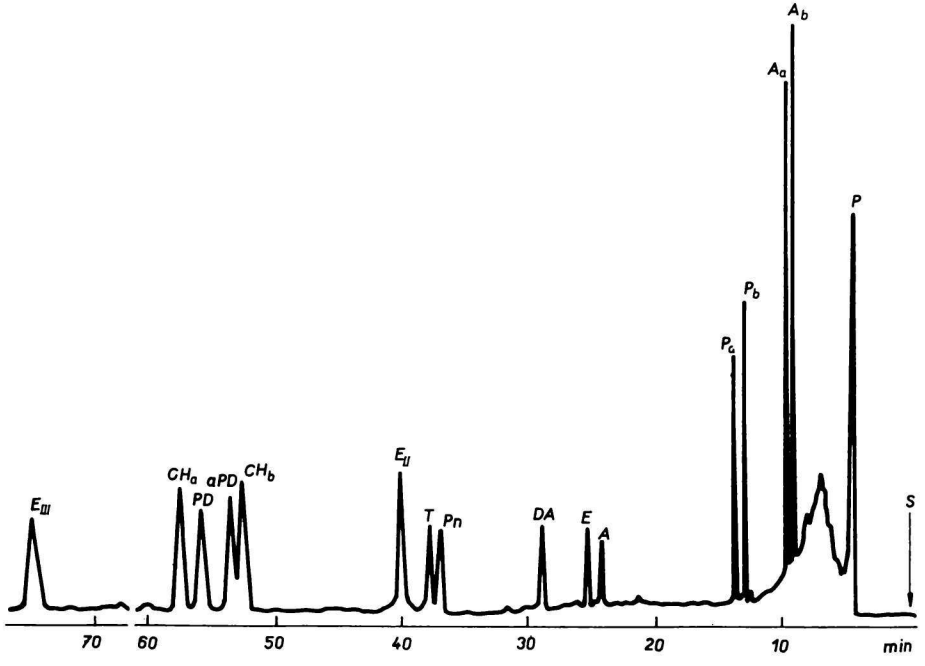

Fig. 2. Separation of the model mixture of steroid hydrocarbons and TMS derivatives of steroids on OV-101 phase at 503 K.
S — start; P — pentane.

isomers is reversed, *e.g.* androsterone before etiocholanolone and allo-pregnanediol before pregnanediol.

For MO—TMS derivatives, at 503 K, for the same column the elution order of the geometrical isomers is the same as for TMS derivatives (Fig. 3).

The MO—TMS derivatives of some keto steroids may occur in two forms as *syn* and *anti* isomers.

Horning et al. [16] published the separation of the *syn* and *anti* forms of testosterone on a polar (OV-17) phase. For the MO—TMS derivative of testosterone separated on a non-polar phase (OV-101) we have found two peaks. Both peaks gave identical mass spectra so that keto-enol isomerism has been excluded.

The influence of temperature on the separation of the TMS derivatives of pregnanolone, testosterone, and estradiol is demonstrated in Fig. 4. In Fig. 5 the

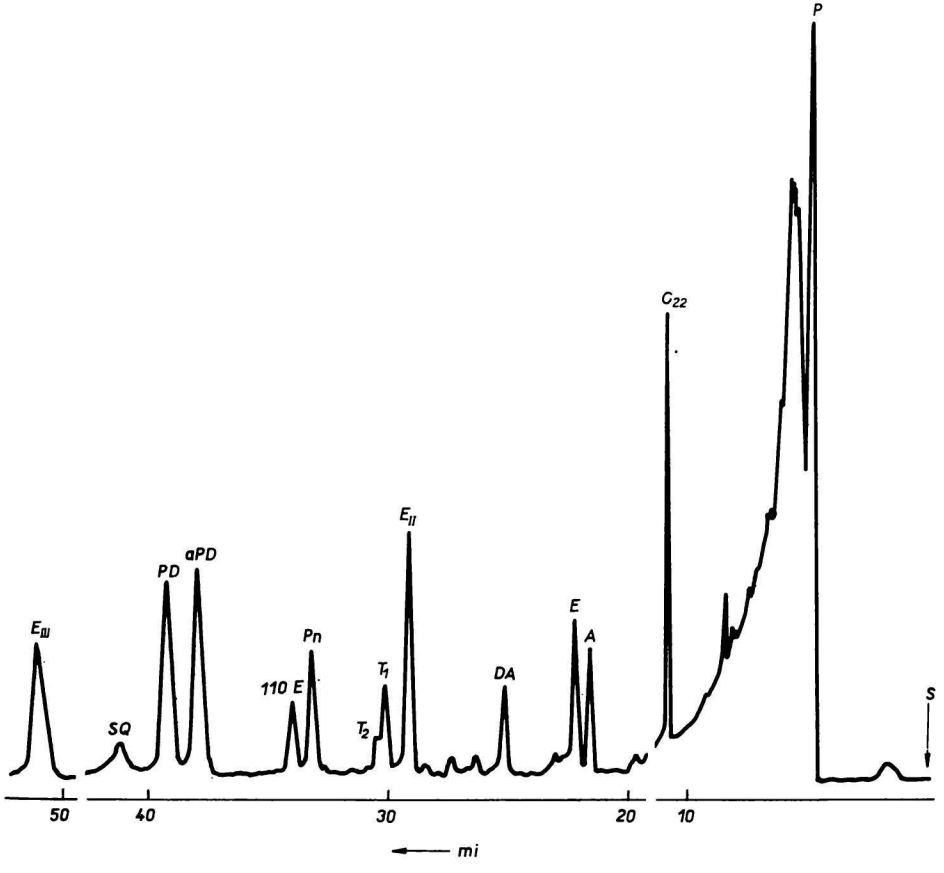

Fig. 3. Separation of the TMS and MO—TMS derivatives of some steroids on OV-101 phase at 503 K. S — start; P — pentane.

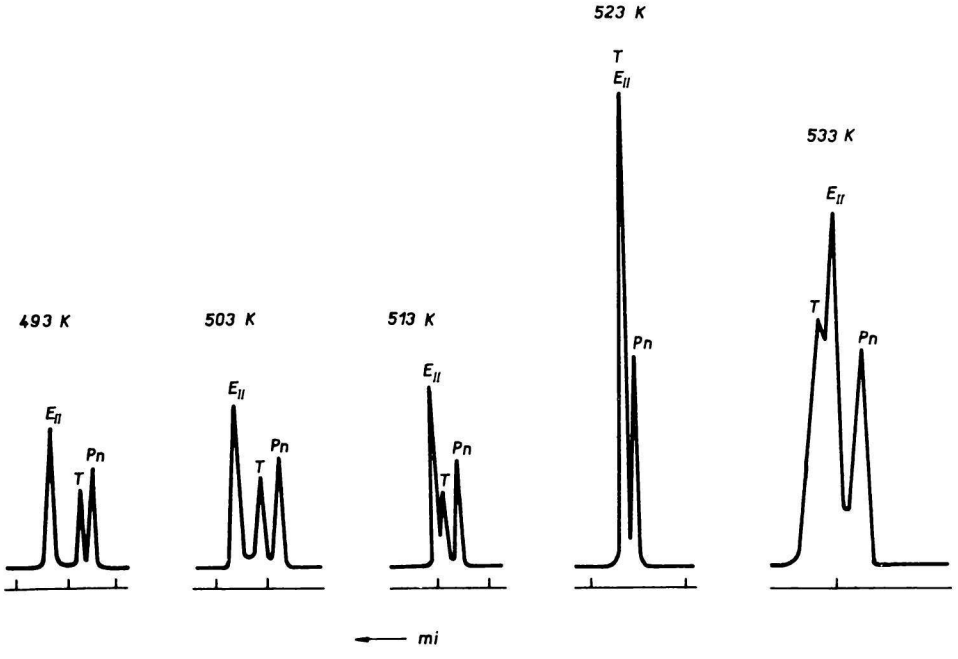

Fig. 4. Influence of temperature on the separation of the TMS derivatives of pregnanolone, testosterone, and estradiol on OV-101 phase.

separation of the TMS derivatives of pregnanediol, allo-pregnanediol, and 5α- and 5β-cholestanes and octacosane is shown. Squalene noticed at 493 and 513 K as an impurity, was identified by mass spectrometry. Its injection is not reproducible and it can considerably affect the retention data of 5α-cholestane and octacosane, often used as standards.

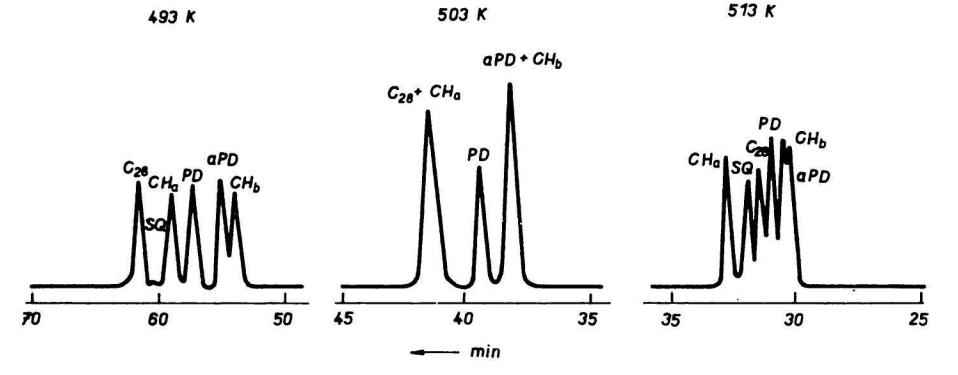

Fig. 5. Influence of temperature on the separation of the TMS derivatives of pregnanediol, allo-pregnanediol, and 5α-, 5β-cholestanes and octacosane on OV-101 phase.

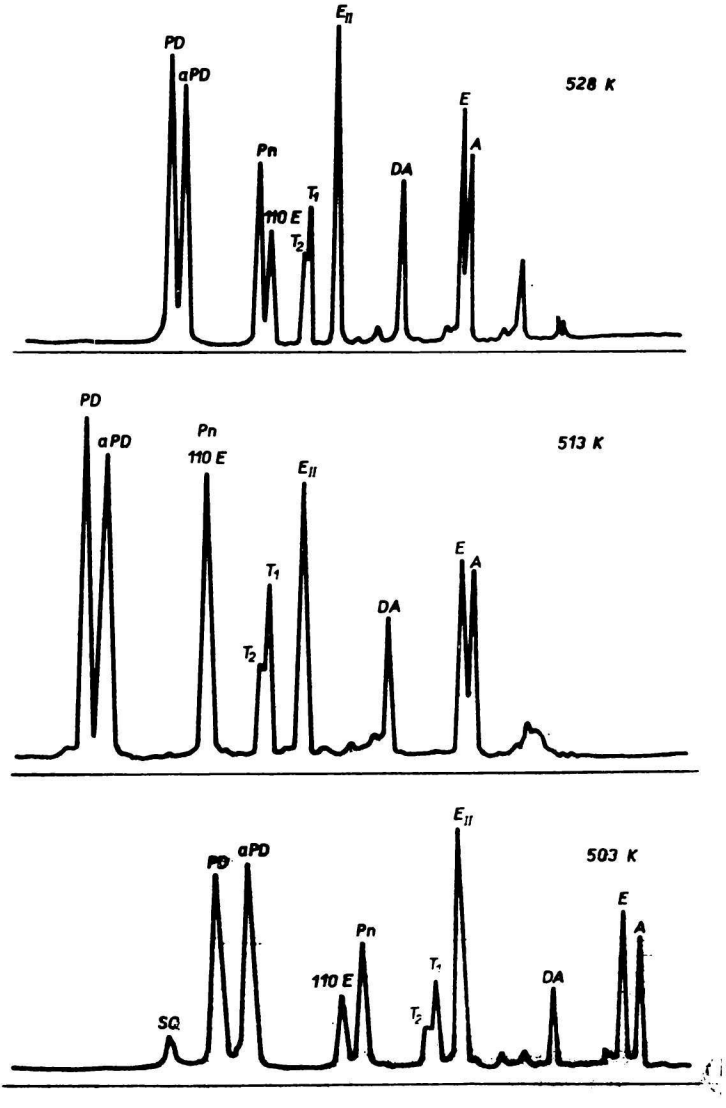

Fig. 6. Influence of temperature on the separation of the TMS and MO—TMS derivatives of steroids on OV-101 phase.

The influence of temperature on the separation of TMS derivatives of steroids and MO—TMS derivatives of keto steroids is demonstrated in Fig. 6. The $\partial I/\partial T$ values for MO—TMS derivatives of keto steroids are smaller than those for TMS derivatives as can be concluded from Table 3 and Figs. 4 and 6.

The Kováts retention indices (I) and the temperature gradients ($\partial I/\partial T$) of some steroid hydrocarbons, TMS derivatives of steroids and MO—TMS derivatives of

Table 3

Kováts indices of TMS derivatives of some steroids measured on different columns coated with OV-101 phase at 503 K

Compound	Column *A*			Column *B*		
	I	σ	*n*	*I*	σ	*n*
Androsterone	2484.4	0.1	4	2485.6	0.2	4
Etiocholanolone	2499.5	0.1	4	2500.5	0.2	4
Dehydroepiandrosterone	2551.6	0.1	4	2552.8	0.2	4
Pregnanolone	2641.8	0.1	4	2643.0	0.2	4
Estradiol	2667.1	0.1	4	2668.2	0.2	4
Allo-pregnanediol	2770.1	0.2	4	2769.1	0.2	4
Pregnanediol	2781.7	0.2	4	2782.7	0.2	4
Estriol	2882.5	0.2	4	2883.3	0.2	4

Table 4

Kováts indices and their temperature gradients of steroid hydrocarbons, TMS and MO—TMS derivatives of some steroids measured on column *A*

Hydrocarbons	513.6 K	524.2 K	$\partial I/\partial T$
5β-Androstane	2051.3	2068.1	1.59
5α-Androstane	2082.4	2100.2	1.68
5β-Pregnane	2237.2	2254.9	1.67
5α-Pregnane	2266.9	2285.5	1.75
5β-Cholestane	2786.1	2804.3	1.72
5α-Cholestane	2819.3	2837.8	1.75
TMS derivatives of:			
Androsterone	2499.4	2514.7	1.44
Etiocholanolone	2513.0	2526.8	1.30
Dehydroepiandrosterone	2567.6	2583.9	1.54
Pregnanolone	2658.2	2675.0	1.58
Testosterone	2670.6	2690.6	1.94
Estradiol	2679.1	2691.3	1.15
Allo-pregnanediol	2782.7	2795.5	1.21
Pregnanediol	2793.6	2805.7	1.14
Estriol	2891.7	2901.0	0.88
MO—TMS derivatives of:			
Androsterone	2558.1	2570.5	1.17
Etiocholanolone	2568.4	2578.9	1.00
Dehydroepiandrosterone	2622.1	2634.8	1.20
Testosterone*	2696.1	2712.0	1.50
Pregnanolone	2732.4	2746.7	1.35

* Values for the first peak of MO—TMS derivative of testosterone (see Fig. 6).

keto steroids are listed in Table 4. The Kováts indices of MO—TMS derivatives of keto steroids are greater than the corresponding values for TMS derivatives. The $\partial I/\partial T$ values are greater for TMS derivatives. The $\partial I/\partial T$ values of TMS derivatives of keto steroids are greater than those for hydroxy steroids.

Conclusion

The reproducibility of the measurement of retention data for the characterization of steroids can be considerably improved by a better temperature and pressure control and the use of high efficiency glass capillary columns. In this way additional information can be obtained on the identity of a component from the temperature gradient of retention indices ($\partial I/\partial T$) that depends on the nature of the substituents, their position in the skelet, and on the configuration of the molecules.

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