Steroids: GC Analysis

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Abstract
Insufficient volatility and thermal stability are the biggest challenges in the application of gas chromatography for the analysis of steroids. The development of gas chromatography (GC) methods for steroids analysis should take in consideration thermal stability, the derivatization step to increase volatility, the stationary phase, and the detector. Several GC methods have been summarized for the analysis of various steroids including anabolic steroids, brassinosteroids, corticoids, estrogen, endocrine-disrupting chemicals, neurosteroids, sterols, steroid saponins, and steroid alkaloids.

INTRODUCTION

Steroids are a class of compounds that have a cyclopentanoperhydrophenanthrene skeleton, which occur in nature and in synthetic products, and which can be classified into six groups according to the number of C atoms: gonane (C-17), estrane (C-18), androstane (C-19), pregnane (C-21), cholane (C-24), and cholestane (C-27). These compounds, except for cholane, are natural hormones or hormone precursors. In the naturally occurring steroids, the fusion of rings B and C is always *trans* and of the rings C and D usually *trans* (*cis* in cardenolides and bufadienolides). Rings A and B are fused in *cis* and *trans* configurations with about equal frequency. Natural steroids possess either one or, more usually, two methyl groups at angular positions at which two rings meet. According to their function, steroid hormones can be divided into estrogens, androgens, gestagens, and corticoids.

The other steroids such as bile acids (cholane), vitamin D, saponin steroids, steroid alkaloids, cardiac glycosides, and brassinosteroids also have biologically important activities. Owing to the metabolic versatility of steroid molecules, extremely complex mixtures are often encountered, necessitating the use of chromatographic methods like high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC), and gas chromatography (GC) for their analyses.

The application of GC to steroid analysis seems to face many difficulties owing to the insufficient volatility and thermolability of the steroids. The development of high-resolution gas chromatography (HRGC) and various derivatization procedures enables the efficient separation of complex steroid mixtures for application in clinical- and forensic toxicology and natural product analysis. The development of low-cost mass spectrometry (MS) detectors in recent years has also promoted the application of GC/MS systems for the analysis of complex mixtures.

THERMAL STABILITY OF STEROIDS

According to their thermal stability, steroids can be divided into three groups.[1] The first group of steroids can be analyzed by GC without any difficulties. This includes steroids that possess Δ^5-3-hydroxy, Δ^4-3-keto, $\Delta^{1,4}$-3-keto, 11-hydroxy, 17-hydroxy groups, and the phenolic ring A in free, ether, or ester form. The steroids of the second group possess tertiary hydroxyl groups (e.g., 17α-methyl-17-hydroxy steriods) and involve de-ethynlation of 17α-ethynyl-17-hydroxy steroids to 17-ketone. These steroids undergo a certain decomposition at high temperatures, but this decomposition could be suppressed by a careful selection of te GC experimental procedures. The third group of steroids decomposes during analysis by GC; hence, their direct chromatographic determination by GC cannot be carried out. The steroids belonging to this group are corticosteroids and Δ^4-3-hydroxy or acyloxy derivatives. Another source of instability is the possible decomposition of steroids and their derivatives by metals; so, using all-glass systems, including glass-line vaporizer of the GC equipment, is essential.[2]

DERIVATIZATION OF STEROIDS FOR GC ANALYSIS

The main objectives for the derivatization of steroids are to decrease heat sensitivity; avoid irreversible adsorption onto the stationary phase; increase volatility; increase the separation efficiency; achieve enhanced selectivity of separations,

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and improve the sensitivity of the detectors.[2] Segura, Ventura, and Jurado[3] described some important general requirements for derivatization reactions: a single derivative should be formed for each analyte; the reaction should be simple and rapid, and they should occur under mild conditions; the derivate should be stable, reproducible, and produced with high yield; in quantitative analyses, the calibration curve should be linear. Different derivatization reagents in combination with catalyzers or antioxidants have been reported in literature.[4,5] In most cases, trimethylsilylation (TMS) derivatization is preferred using *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) as the derivatization reagent.[4,5] A mixture of MSTFA and trimethyliodosilane (TMIS), which is usually called $MSTFA^{++}$, can also be used as the derivatization reagent before GC–MS/MS analysis.[4] Other derivatization reagents, e.g., acetic acid anhydride, trifluoroacetic anhydride (TFA), pentafluoropropionic acid (PFPA), or heptafluorobutyric acid (HFBA) anhydride, can also be used.[3,6] For anabolic steroids and metabolites containing nitrogen, the use of acyl derivatives is also interesting.[3] The primary factor upon which the choice of a suitable derivative will be dependent is the type of detector used. For the application of the highly sensitive electron capture detector (ECD), it is quite favorable to prepare derivatives that show a high affinity for electrons; so, in such cases, chloroacetate and heptafluorobutirate derivatives are mostly used. For analysis of steroids using isotope dilution GC/MS (GC–ID/MS), silylation is not recommended on account of the fairly high abundance of ^{29}Si and ^{30}Si in natural silicon,[6] so derivatization using TFA, PFPA, or HFBA is preferred. Enzymatic hydrolysis is required before performing derivatization of glucono- or sulfoconjugated steroids in urine.[7,8] Derivatization of steroids using solid-phase microextraction (SPME) and head space methods was also described previously.[9] Conventional derivatization procedures used conventional heating process in an oven, sand bath or water bath, which is very time-consuming. Recently Zuo, Zhang, and Lin[10] reported the use of microwave in the formation of trimethylsilyl derivatives of estrogen steroids for GC–MS analysis. The advantage of this method is the minimization of the time needed for the derivatization process of estrogenic steroids. Also, the derivatives produced using microwave acceleration were identical and comparable to those produced by the conventional heating method. Detailed discussions of the derivatization methods for steroids are provided in an earlier entry in this encyclopedia[11] and in Refs. 3, 10.

STATIONARY PHASES

In the analysis of steroids by GC, silicone oils (SE-30, OV-1, OV-101) are most often used. These phases are suitable for the analysis of steroids on the basis of their molecular weight or the shape of the molecules. These silicone phases are considered as non-selective stationary phases. For separating stereoisomers or structural isomers, saturated or unsaturated derivatives, selective stationary phases such as methyltrifluoropropyl (QF-1, OV-210), methylphenylcyanopropyl (OV-225), and methyl-β-cyanoethyl (XE-60, AN-600) can be used.[2] For packed-column applications, solid supports such as Gas-Chrom Q, Gas-Chrom S, Chromosorb AW or DMCS, and Chromosorb W HP DMCS, with 3% concentration of the stationary phase, have been generally used.[1,12] For separating complex steroid mixtures, application of a glass or a fused-silica column is recommended. The length of the column is in the range between 17 and 30 m, diameter of 0.2–32 mm, and film thickness of 0.1–0.33 μm. The stationary phase consists mostly of polydimethylsiloxane, with 0–50% diphenyl groups.[6]

DETECTORS

The flame ionization detector (FID) is the detector most often used in steroid analyses. For very low concentrations of steroids, the application of ECD is needed. Thermal conductivity detectors (TCDs) cannot be used in the analysis of steroids because of their very low sensitivity.[1,2] For steroidal alkaloids, a nitrogen-specific detector (NPD) has also been used. By the use of dual detector systems (e.g., FID and NPD), closely related nitrogen-containing and non-nitrogen-containing steroids can be easily differentiated. The application of MS as detector was already discussed in a previous entry in this encyclopedia.[13] By using a GC/MS system, the identity of the peak(s) can be determined in an undisputed manner.[3]

GC PARAMETERS FOR STEROID IDENTIFICATION

The identification of steroids in an unknown sample can be based on GC or GC/MS parameters, such as relative retention times, retention indices, steroid number, mass spectra, and/or important ion fragments.

Relative retention time (RR_t) is defined as the ratio of the analyte's and the reference compound's retentions. The RR_t of sterols relative to cholesterol and cholestane, stearyl acetates to cholesteryl acetate, estrogen derivatives to cholestane, and estrogen TMS ether to cholestane, on various packed columns, was reported by Heftmann.[12] The RR_t of some estrane, androstane, and pregnane derivatives was also reported.[2]

The concept of retention index (RI) was first published by E. Kovats in 1961. Detailed discussion on RI is included in a previous entry in this encyclopedia.[14] Van Gelder[15] described the RI of various steroid alkaloids and saponins.

Steroid number (SN) is the sum of quantities characteristic of the skeleton and the functional groups in a molecule.[1,9]

$$SN = S + F_1 + F_2 + \ldots + F_N$$

where S is the number of C atoms, and F_1, F_2, ..., F_N are values that are characteristic of the functional groups. The values of SN and F of some steroids, determined in packed columns, were already described in previous works.[1,12]

GC/MS parameters for anabolic steroid metabolites,[7,16,17] steroid alkaloids, and saponins,[15] and their MS data have been published previously.

SELECTED APPLICATIONS

Anabolic Steroids

Anabolic steroids, which are related in structure and activity to testosterone, are used to improve muscle mass and to accelerate recovery times from exercises. The use of anabolic steroids by athletes during competition and training was forbidden by the International Olympic Committee (IOC). Anabolic steroids have been abused not only by humans, but also for sporting purposes such as horse racing.[18,19,20] For doping control in sports, urine samples are mostly tested. The most often used anabolic steroids are androgens, such as testosterone.[4] It is not easy to detect parent compounds except for oxandrolone and testosterone, because the steroids are extensively metabolized.[21] Before derivatization, urine samples are passed through a Pasteur pipette containing Serdolit AD-2 slurry[21,22] or extracted using solid-phase extraction (SPE).[7,8] For conjugated steroids, hydrolysis is performed (at pH 8.5) before derivatization.[8] For analysis of anabolic steroids, Choi et al.[21] used an Ultra-2 cross-linked capillary column of 5% phenylmethylpolysiloxane (30 m × 0.2 mm, film thickness 0.33 μm) with an oven temperature of 150°C (2 min) to 300°C (2 min) with a step rate of 20°C/min; injection temperature of 300°C; and detection using MS (negative chemical ionization using methane) and tandem MS (MS/MS with collision energy for a collision-induced dissociation of 1.0). Yoon and Lee[8] used a glass capillary column (17 m × 2 mm) with an oven temperature of 180°C (6 min), then increased to 224°C (4°C/min), and finally to 300°C (15°C/min), and detection using MS (negative chemical ionization using methane). Ho et al.[19,20] reported the analysis of turinabol and mesterolone in horse urine using an HP1-MS column (~30 m × 0.25 mm, 0.25 μm film thickness) with constant helium flow at 1.2 ml/min. For the analysis of turinabol the oven temperature was set at 60°C (1 min), then increased to 220°C (60°C/min) and, finally, to 300°C (3°C/min), which was held for 5 min, while for mesterolone the column was initially set at a higher temperature of 120°C (0.5 min), then increased to 180°C (30°C/min), followed by an increase to 230°C (4°C/min), and finally to 300°C (30°C/min), which was held for 5 min. The MS analysis was performed on electron impact (EI) mode. The analysis of residual anabolic steroids in meat was reported by Fuh, Huang, and Lin.[23] The isolation was conducted by SPE. To derivatize the isolated steroids, $MSTFA^{++}$ was used. The enzymatic hydrolysis was not conducted, as previous studies have discovered that there was no significant hormone liberation involved in muscle, fatty tissue, and meat. The derivatization products were then subjected to GC–MS/MS analysis. For this purpose, a DB-5 GC column (30 m × 0.25 mm, with 0.25 μm film thickness) was used, with a flow rate of 1.0 ml/min. The oven temperature was initially set at 180°C (1 min), increased to 240°C (6°C/min, for 2 min), and finally increased to 290°C (6°C/min, held for 10 min). The MS analysis was performed on EI mode. Impens et al.[24] used a non-polar 5% phenyl-polysilphenylene-siloxane BPX-5 GC column to analyze anabolic steroids in bovine urine. The sample was derivatized with $MSTFA^{++}$ before being injected for the GC–MS/MS and GC–MS/MS/MS analyses. The oven temperature was initially set to 100°C (1 min), then increased to 250°C (30°C/min), followed by an increase to 290°C (2.5°C/min), and finally to 300°C (10°C/min), which was held for 1.5 min. Interested readers can consult Refs. 3, 8, and 18–25 for details.

Brassinosteroids

Brassinosteroids are steroidal plant hormones that are required for normal growth and development. Brassinosteroids are distributed in both aerial and underground parts of plants; however, the concentration is higher in the aerial parts.[26] About 40 brassinosteroids have been identified from the plant kingdom.[27] The use of brassinosteroids as an anticancer agent has been investigated by Malíková et al.[28] For the analysis of brassinosteroids, Park, Kim, and Kim,[27] Kim et al.,[29] and Kim, Kim, and Kim[26] used an HP-5 fused-silica column (30 m × 0.25 mm, film thickness 0.25 μm) with an oven temperature of 175°C (2 min), then elevated to 280°C (40°C/min). Prior to injection, the samples were treated with methaneboronic acid in pyridine (70°C for 30 min) to produce bismethaneboronate. For analyzing typhasterol, testosterone, 6-deoxytyphasterol, and 6-deoxoteasterone, Nomura et al.[30] silylated the methaneboronate to MB-TMS derivatives before injecting into the GC (capillary DB-1 column 25 m × 0.25 mm, 0.25 μm film thickness; oven temperature 170°C for 1.5 min, then increased to 280°C in steps of 37°C/min).

Corticoids

Corticoids or corticosteroids are divided into mineralocorticoids that act in the regulation of blood volume and metabolism of electrolytes, and glucocorticoids that act in saccharometabolism.[4,25] On account of the instability of

their dihydroxy acetone side chain at C-17, direct analysis using GC is impossible. Methoximation followed by TMS derivatization is the most widely used approach.[3] For the analysis of glucocorticoids, Mozzarino et al.[31] used an HP-1 fused-silica cross-linked methyl silicone capillary column (17 m × 0.20 mm, film thickness 0.11 μm) at a flow rate of 1 ml/min. The temperature was set at 180°C (4.5 min), increased to 230°C (3°C/min), followed by an increase to 290°C (20°C/min), and finally to 320°C (30°C/min). The derivatization with MSTFA/NH_4I/dithioerythrytol (1000:2:4, v/w/w) at 70°C for 20 min was conducted before GC–MS analysis. Shakerdi et al.[32] investigated a GC–MS method to analyze the excretion rate of 18-hydroxytetrahydro-11-dehydrocorticosterone using the non-homologous reference standard β-cortol. After derivatization the sample was injected to a DB-1 column (30 m × 0.322 mm, film thickness 0.25 μm). The oven temperature program was as follows: starting from 100°C (3 min) increased to 190°C at a rate of 20°C/min, then to 285°C at a rate of 2°C/min, which was held for 10 min. The analysis was performed on positive EI ionization mode. Interested readers should consult previous publications.[6,25,31,32]

Estrogens

Estrogens (C18) are regarded as typical female hormones owing to their importance in the estrous cycle. These hormones have been used in animal fattening because of their anabolic effects. These C18 steroids differ from all other steroids in a way characterized by the presence of an aromatic A ring and the lack of a methyl group at C-10. Fritsche[33] analyzed 17β-estradiol and estrone and the estrogenic metabolites estriol and 17β-estradiol in foodstuffs using a DB-5 fused-silica column (30 m × 0.25 mm, film thickness 0.25 μm) and detection using MS (EI, electron energy: 60 eV, ion source temperature: 180°C). The steroids were previously derivatized with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide/TMIS/dithioerythrytol (1000:2:2) at 60°C for 15 min. Roy, Hachey, and Liehr[34] developed a GC method with a ^{53}Ni-pulsed ECD for measuring the rate of formation of 2-hydroxyestradiol and 4-hydroxyestradiol from estradiol in microsomal preparations. The steroids were converted to heptafluorobutyryl esters and separated with a DB-5 fused-silica capillary column (30 m × 0.25 mm). The GC conditions were splitless injection, 280°C injection temperature, and a temperature gradient of 30°C/min from 100°C to 245°C followed by a 5 min isothermal period at 245°C and a second temperature gradient of 1°C/min from 245°C to 265°C. GC analysis of the pentafluorobenzyl derivatives of estrogen in river water and effluents was also developed by Xiao, McCalley, and McEvoy.[35] Impens et al.[36] have undertaken a study on the residues of estrogens in kidney fat and meat. For this purpose the sample was derivatized with MSTFA, which was then subjected to GC–MS analysis. For this purpose, a non-polar 5% phenyl-polysilphenylene-siloxane SGE BPX-5 column (25 m × 0.22 mm, film thickness 0.25 μm) was used. The initial oven temperature was keep at 100°C for 1 min, then increased to 250°C at a rate of 30°C/min, then to 290°C (2.5°C/min), and finally to 300°C (10°C/min), which was held for 1.5 min. For further confirmation of the steroids a GC–MS/MS analysis was performed.

ENDOCRINE-DISRUPTING CHEMICALS

Endocrine-disrupting chemicals (EDCs) are exogenous substances that cause alterations of normal hormone function and physiological status in wildlife and in humans.[37,38] There are two major types of compounds classified as EDCs: phenolic and estrogenic. These dangerous chemicals can be released directly or indirectly from domestic or industrial waste as pollutants in aquatic environment. Analysis of EDCs in sludge and waste water samples have been described by Jeannot et al.[38] The samples were extracted by SPE with C18 cartridges and SPE with polymeric cartridges. Before GC–MS and GC–MS/MS analyses, the sample was derivatized with bis(trimethylsilyl)trifluoroacetamide (BSTFA). A CP-Sil 8 CB column (95% dimethyl-5% phenyl polysiloxane, 30 m × 0.25 mm, film thickness 0.25 μm) was used. The oven temperature was programmed at 85°C (3 min), then increased to 130°C (10°C/min), and then slowly to 300°C (3°C/min). Liu, Zhou, and Wilding[37] have developed a simultaneous analysis of EDCs in water samples using SPE–GC–MS method. For this purpose nine types of SPE cartridges were employed. The sample derivatization was carried out with BSTFA at 60–70°C for 30 min same as Jeannot et al.[38] The GC–MS analysis was conducted on a ZB5 (5% diphenyl-95% dimethyl polysiloxane) capillary column (30 m × 0.25 mm, film thickness 0.25 μm). The column temperature was initially set at 100°C (1 min), which was then increased to 200°C (10°C/min), followed at a rate of 15°C/min until it reached 260°C, and finally slowly increased at a rate of 3°C/min to 300°C, which was held for 2 min. Interested readers should consult Refs. 37 and 38.

Neurosteroids

Neurosteroids are steroids that are synthesized de novo in the central nervous system (androsterone, dihydrotestosterone, testosterone, allopregnanolone, isopregnanolone, and pregnanole). These steroids are synthesized from cholesterol or from a blood-borne precursor.[39] After the extraction of plasma and cerebrospinal fluids using SPE and derivatization with carboxymethoxime, pentafluorobenzyl, and trimethylsilyl, the derivatized samples were injected into a GC/MS system for quantitative evaluation with a selected-ion monitoring (SIM) method, which was used to maximize sensitivity as only a selected *m/z* is monitored. Details of the method have been described in Refs. 40 and 41. To analyze neurosteroids in the rat brain, Liere et al.[42,43] used a BPX5 column (5% phenyl-95% dimethyl polysiloxane, 25 m × 0.22 mm, film thickness

0.25 μm) and a BPX35 column (35% phenyl-65% dimethyl polysiloxane, 30 m × 0.25 mm, film thickness 0.25 μm). The oven temperature was set as follows: starting at 50°C (1 min), increased to 140°C (30°C/min), followed by an increase to 300°C (10°C/min) for the first column; and starting at 50°C (1 min), increased to 175°C at the rate of 30°C/min, and finally to 320°C at the rate of 10°C/min for the second column. The MS was performed in the EI mode. Before being injected to GC–MS, samples were derivatized with HFBA anhydride or triethylamine (TEA)/HFBA. A review article on the analysis of neurosterols and neurosteroids by MS has been published by Wang, Karu, and Griffiths.[41]

Sterols

Various TMS derivatives of sterols in tuna olive oil could be separated using CP-Sil 8 CB fused-silica column (15 m × 0.22 mm; 240°C isothermal; FID). Plant sterols (brassicasterol, campesterol, stigmasterol, and sitosterol) were analyzed using a fused-silica column (15 m × 0.25 mm, 260°C isothermal; FID).[44] The analysis of campesterol, sitosterol, and diosgenin using a glass-packed column (6 ft × 0.125 in.; 3% SE-30 on Gas Chrom Q, 220–270°C, 4°C/min; FID) was described.[45] The separation of plant sterols (cholesterol, campesterol, stigmasterol, and sitosterol), squalene, and some lupane triterpenes using a glass-packed column (2 m × 2 mm; 3% OV-1 on Gas Chrom Q; 200–280°C, 4°C/min; FID) was reported.[46] The analysis of sterols (β-sitosterol, stigmasterol, campesterol, and cholesterol) in sugarcane waxes was undertaken in a fused-silica capillary column, CP Sil 5 (25 m × 0.25 mm, film thickness 0.25 μm). The temperature was programmed from 150°C to 320°C at the rate of 6°C/min. The MS was recorded in EI mode. TMSi derivatives of sterols were prepared using HMDS/TMSiCl (NF ISO 6799).[47] Seo et al.[48] reported the analysis of squalene and phytosterol (β-sitosterol and stigmasterol) in *Eleutherococcus senticosus* using an HP-1 (25 m × 0.25 mm, film thickness 0.33 μm) methyl polysiloxane cross-linked capillary column. The temperature was programmed from 100°C to 250°C at the rate of 20°C/min. The amount of squalene and phytosterol was calculated from the ratio of the peak area of the relevant compound to that of the standard. To analyze the constituents (alkanes, esters, aldehydes, ketones, fatty acids, and sterols) of cuticular waxes from potato, GC–MS analysis was performed in a RTX-1 WCOT capillary column (30 m × 0.25 mm, film thickness 0.25 μm). The oven temperature was initially set to 200°C and then increased to 320°C (4°C/min), which was held for 15 min.[49] The analysis of phytosterol in fungus *Mortierella alpine* was conducted in a DB-5 column (30 m × 25 mm, film thickness 0.25 μm).[50] Analysis of plant sterols in food and vegetable oils has been reviewed by Abidi.[51] Kalo and Kuranne[52] described the GC analysis and electrospray tandem MS of free sterols in fats and oils.

Steroid Saponins and Steroid Alkaloids

Steroidal saponins are steroids coupled to sugar units. Before GC analysis, steroid saponins need to be derivatized to their acetyl, methyl, or trimethylsilyl ether derivatives. There are two methods that can be used to analyze steroidal saponins using GC. First, GC analysis was conducted on the steroidal saponins after the separation of aglycone and sugar moiety through hydrolysis. Second, the analysis was undertaken in steroidal saponins without hydrolysis of the sugar units. The latter method can be used if the sugar units are not attached to the steroids through ester linkage; otherwise, the deglycosylation of the steroidal saponins can occur in the injection port of the GC.[53]

The separation of diosgenin and solasodine was reported using a glass-packed column (6 ft × 0.125 in.; 3% SE-30 on Gas Chrom Q, 220–270°C, 4°C/min; FID). Diosgenin and its 5-α-derivative (tigogenin) can be separated well after derivatization using TFA, and analyzed using a glass-packed column (10 ft × 0.125 in.; 3% QF-1 on Gas Chrom Q; 220°C isothermal; FID).[45] Solanidine, demissidine (5-α-derivative of solanidine), and solasodiene, which could not be separated using a glass-packed column (1 m × 2 mm; 10% SE-30 on Chromosorb W-HP, 260–300°C, 5°C/min, FID), were separated well using a fused-silica column (50 m × 0.22 mm; CP-Sil 5, film thickness 0.12 μm; 290°C isothermal; FID).[13] Using a fused-silica column (50 m × 0.22 mm; CP-Sil 5 CB, film thickness 0.12 μm; 270°C isothermal; FID), various steroids, 5-α-cholestane, solanthrene, cholesterol, solanidine, demissidine, solasodiene, stigmasterol, diosgenin, tigogenin, solasodine, and tomatidine, could be separated without derivatization.[15] Some methods of analysis of solasodine using GC were described in a previous publication.[54]

Li et al.[55] has developed a method to analyze isosteroidal alkaloids (ebeiedine, ebeiedinone, ebeienine, hupehenine, isoverticine, verticine, verticinone, and imperialine) in *Fritillaria* species through direct injection of these alkaloids without precolumn derivatization. Two GC columns, namely Supelco SAC-5 (30 m × 0.25 mm, film thickness 0.25 μm) and HP-1 (12.5 m × 0.22 mm, film thickness 0.33 μm) were compared. The result has shown that in Supelco SAC-5 six out of the eight alkaloids tested were resolved better.

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