

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá D.C.,

Abogado Mauricio Álvarez Gómez
Carrera 1a. No. 22 – 58, Of 407, Santa Marta

ASUNTO	Radicación	09 126 428
	Trámite	002
	Evento	001
	Actuación	519
	Oficio	
	Folios	53, 54, 55 - 17 8 1 5

Patente de Invención	☐	Patente de Modelo de Utilidad	☐
Vía Nacional	☐		

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 123 a 226	24/Agosto/2007
Reivindicaciones:	Folios 227 a 230	24/Agosto/2007

2. Objeto de la invención

Título de la solicitud: "Método de determinación de metanol en glicerina por cromatografía de gas con sistema Head Space".

El problema planteado en esta solicitud es suministrar un método para la determinación de metanol en glicerina donde no represente ensayos y equipos más costosos.

Para resolver este problema técnico la solicitud en estudio proporciona un método de análisis para la determinación del contenido en porcentaje másico de metanol en glicerol, proveniente del proceso de fabricación de biodiesel, aplicando cromatografía de gases con head space.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): G01N 30/00.

3. De la Solicitud de Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las reivindicaciones no se encuentran debidamente sustentadas en la descripción e ilustradas, puesto que no se mencionan ensayos específicos de la determinación de alguna o algunas muestras, donde la preparación de la muestra posiblemente varié o quizás sea necesario un tratamiento previo o sea necesario cambiar la columna o tal vez el solvente¹, puesto que "la preparación de la muestra es una etapa decisiva en todo método de análisis en especial en la determinación de microcomponentes (trazas) y en los casos donde la matriz que rodea el analito es muy compleja. La selección del método más apropiado

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depende de muchos factores como ser: propiedades físicas y químicas del analito, concentración de analito en la muestra, naturaleza de la matriz de la muestra, forma en que se presenta el analito en la muestra, compatibilidad de los medios de solubilización y extracción con el sistema cromatográfico, tipo de detector, compatibilidad con el detector¹. El solicitante debe definir específicamente la materia y el alcance de la invención a través de las características técnicas esenciales que se encuentren enteramente soportadas en la descripción.

Las características técnicas esenciales del método definido en el capítulo reivindicatorio pertenecen a una determinación de un analito (metanol) en una muestra antes no analizada (glicerina) en donde lo que se hace comúnmente es una revalidación del método para las condiciones del equipo y de la muestra que se tienen².

4. Determinación del Estado de la Técnica

El Estado de la Técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: Noviembre 09 de 2009, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación*
D1	PerkinElmer, ecoanalytix	Biodiesel Glycerin and Methanol Analyzer	2008
D2	Thermo Scientific Mumari F. et Al	Determination of Methanol Content in Pure Biodiesel (B100) by Headspace-GC in Compliance with EN14110	2007
D3	Agilent Technologies, Application Note SI-0773, Coen Duvekot, Varian, Inc.	Determination of Methanol Content in Biodiesel using Gas Chromatography with Headspace Sampling According to EN-14110	2008
D4	Maria C, et Al, Bioresource Technology, vol 99, pages 5901 - 5905	Determination of methanol by headspace solid phase microextraction	26 Noviembre 2007

D1 divulga (folios 48 – 51) que el PerkinElmer ® EcoAnalytix TM Biodiesel Glicerina y el Analizador de metanol proporciona una solución única para poner a prueba el biodiesel mediante el método GC incluidas tanto en el EN y ASTM. El analizador incorpora un TurboMatrix TM Headspace Sampler acoplado a un cromatógrafo de gases Clarus ®. El Biodiesel Glicerina y el Analizador de metanol consiste de tres componentes principales: el espacio de cabeza TurboMatrix Sampler, el GC Clarus, y un sistema integrado auxiliar de una Estufa, isoterma en el GC. El Cromatógrafo de gases Clarus está configurado con un muestreador automático, un inyector programable en columna y dos detectores de ionización de llama.

D2 divulga (folios 52 – 55) un método de determinación del contenido de metanol en biodiesel puro adoptando los métodos estándar que caracterizan un biodiesel, el método empleado utiliza un Thermo Scientific TRACE GC Ultra equipado con un Split/splitless (SSL) de entrada y un detector de ionización de llama (FID), automatizado con un inyector automático TriPlus, configurado para utilizar el espacio de cabeza. La columna analítica era no polar Thermo Scientific TRACE TR – BIODIESEL (M), 30m, 0.32mm de diámetro, 3.0µm. se emplean 3 soluciones de calibración, usando de referencia FAME y agregando metanol puro, donde las concentraciones obtenidas de los patrones fueron de 0.5, 0.1 y 0.01% m/m, luego 2mL de cada solución se transfirieron a viales de espacio de cabeza, los parámetros obtenidos fueron, para el programa en el horno del GC empleado: 50°C (1min) a 130°C, 10°C/min, mantener por 0,5min, la temperatura del detector 250°C y del inyector 160°C, todo para determinar un ontenido de metanol de 0.01% m/m a 0.5%/m/m.

D3 divulga (folios 56 y 57) un método de determinación del contenido de metanol en biodiesel usando cromatografía de gases con espacio de cabeza de acuerdo a la norma EN14-110, donde se empleo un equipo Varian 430-GC:-inyector:Split/splitless 1177, -detector:FID, muestreador de espacio de cabeza-QUMA, QHSS-40 modo muestra loop; como columna una Varian Select TM Biodiesel para metanol, 30m×0.32mm×3.0µm, CP-9083. Se emplearon como soluciones de Expediente 09 126 428 1er Art 45

calibración: -solución A: 0.5% (m/m) metanol en FAME, -solución B: 0.1% (m/m) metanol en FAME, -solución C: 0.01%(m/m) metanol en FAME, luego una alícuota de 1 ml se pesaron con exactitud, y se transfirió a un vial de 20 ml y se tapó inmediatamente. Las condiciones del cromatógrafo de gases:-inyector: 250°C, velocidad Split: 50:1, detector: 275°C, FID, horno: 80°C (0,5min isotérmico), 20°C/min hasta 160°C (2min), gas de corrida: flujo constante de helio 2.0mL/min.

D4 divulga (folios 58 – 61) un estudio en la determinación de metanol en biodiesel por microextracción en fase solida headspace, donde en el método de análisis se menciona que, para a la muestra de biodiesel se le realiza un lavado con agua y se seca toda la noche con Na₂SO₄ anhidro. En la calibración se emplean 6 soluciones de con sus respectivas replicas, donde las concentraciones fueron 0.0057, 0.011, 0.023, 0.034, 0.045, 0.057, 0.11, 0.23, 0.57, 1.14, 1.70, 2.27 % m/m (metanol/biodiesel), se emplea un 1mL de muestra y el programa en el horno fue: temperatura inicial de 40°C por 4min, incrementa a 120°C a 10°C/min y se incrementa a 200°C a 20°C/min, se mantiene a 200°C por 1min, la temperatura del inyector 200°C y la temperatura del detector 300°C, se emplea una columna HP-5 (5%-fenil)-metilpolisiloxano, una relación Split 20:1; finalmente se logra detectar un 0.25% de metanol.

5. Examen de Patentabilidad (Art 14 D486)

Nivel Inventivo (Art18 D486)

La reivindicación 1 consiste en un “método que se caracteriza por, cuantificar el contenido de metanol en glicerina utilizando la técnica Head Space, acoplada a un cromatógrafo de gases”

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2	D3
método	Método	Método	Método
Cuantificar metanol en glicerina	Cuantificar metanol	Cuantificar metanol	Cuantificar metanol
técnica Head Space, acoplada a un cromatógrafo de gases	técnica Head Space, acoplada a un cromatógrafo de gases	técnica Head Space, acoplada a un cromatógrafo de gases	técnica Head Space, acoplada a un cromatógrafo de gases

Los documentos del estado de la técnica cercanos a la invención corresponden a D1, D2 y D3 porque:

D1 divulga que en la producción de biodiésel, ácidos grasos libres (lípidos) convierten catalíticamente en ésteres metílicos de ácidos grasos (FAME) con alcohol, típicamente metanol. Después de este catalizador de la reacción, la glicerina, el agua residual y debe ser eliminado para crear un combustible adecuado para su uso en encendido por compresión (diesel). Un número de problemas de calidad puede surgir si la reacción es incompleta o si los subproductos no se eliminan eficazmente. El PerkinElmer ® EcoAnalytix TM Biodiesel Glicerina y el Analizador de metanol proporciona una solución única para poner a prueba el biodiesel mediante el método GC incluidas tanto en el EN y ASTM. El analizador incorpora un TurboMatrix TM Headspace Sampler acoplado a un cromatógrafo de gases Clarus ®. El Biodiesel Glicerina y el Analizador de metanol consiste de tres componentes principales: el espacio de cabeza TurboMatrix Sampler, el GC Clarus, y un sistema integrado auxiliar de una Estufa, isoterma en el GC. El Cromatógrafo de gases Clarus está configurado con un muestreador automático, un inyector programable en columna y dos detectores de ionización de llama. El horno auxiliar isotérmico instalado dentro de la GC Clarus proporciona una segunda zona de temperatura controlada para una segunda columna cromatográfica. El Muestreador Headspace TurboMatrix permite el análisis desatendido de múltiples muestras (folios 48 - 51, páginas 1 y 2).

D2 divulga que el seguimiento de metanol residual en B100 es una cuestión de seguridad, ya que incluso pequeñas cantidades de este material puede reducir el Expediente 09 126 428 1er Art 45

punto de inflamación del biodiesel. Además, el metanol residual puede afectar a las bombas de combustible, sellos y elastómeros y puede resultar en propiedades de combustión pobre. La norma EN 14110 requiere un método de Cromatografía de gases y de espacio de cabeza, o bien de las columnas polares o no polares, y es aplicable para un intervalo de concentración de 0,01% m/m a 0,5% m/m de metanol (MeOH). El método no es aplicable a mezclas de FAME (ésteres metílicos de ácidos grasos) que contienen otros componentes de bajo punto de ebullición, aunque el uso de la Thermo Scientific TRACE™ TR-BIODIESEL (M) columna permite una buena separación de metanol a partir de la mayoría de estos volátiles, produciendo resultados fiables (folios 52 - 55).

D3 divulga que el rendimiento del FAME (ésteres metílicos de ácidos grasos) puro (una vez que la glicerina y el metanol residual se recuperado/eliminado) se llama B-100. A fin de que el biodiesel pueda ser utilizado como un combustible de motor o mezclado con el diesel de petróleo, debe ser conforme a las especificaciones de la norma (ASTM D 6751 o EN- 14214). Hay métodos de GC usados hoy en día para determinar si el biodiesel se ajusta a las especificaciones de la norma. Uno de estos métodos, EN-14110, se utiliza para determinar el contenido de metanol. EN-14110 es aplicable para un rango de concentración de 0,01% (m/m) a 0,5% (m/m) de metanol. En este trabajo se empleo sistema cromatógrafo de gases Headspace (folios 56 y 57).

La diferencia entre la invención, definida en la reivindicación 1 y el método de D1 consiste en que se hace directamente en biodiesel, mientras que en la presente solicitud se especifica que la muestra es glicerina.

La diferencia entre la invención, definida en la reivindicación 1 y el método de D2 consiste en que se hace directamente en biodiesel, mientras que en la presente solicitud se especifica que la muestra es glicerina.

La diferencia entre la invención, definida en la reivindicación 1 y el método de D3 consiste en que se no se especifica la muestra, mientras que en la presente solicitud se especifica que la muestra es glicerina.

No existe evidencia que el hecho que se especifique que la muestra es glicerina introduzca un efecto técnico inesperado, que represente para la persona de nivel medio versada en la materia una mejor eficiencia en la determinación de metanol, en cuanto a confiabilidad, reproducibilidad y costos.

Por lo tanto, el problema técnico objetivo a resolver es plantear una alternativa al método conocido según D1 o D2 o D3 para mejorar la eficiencia en la determinación de metanol, en cuanto a confiabilidad, reproducibilidad y costos.

Además teniendo en cuenta el estado del arte cuando ya se conoce un método para determinar un analito, se procede a validar el método^{1,2} para que la determinación tenga cierta robustez, precisión y confiabilidad.

En consecuencia, el experto en la materia, teniendo en cuenta las enseñanzas anteriormente mencionadas y las características de los métodos de D1, D2 y D3 podrá llegar sin ningún esfuerzo al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

Las **reivindicaciones 2 – 10** mencionan ciertos parámetros y características cromatográficas del método de determinación de metanol en glicerina (ver folio 19 y 20).

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D2	D3	D4
Método	Método	Método	Método
Obtención de una solución blanco de glicerina libre de metanol, calentar la muestra a 120°C y 769mmHg	Preparación de la muestra, la muestra de referencia FAME se preparar con extracción con agua asegurando el contenido bajo en metanol (0.001% m/m)	No se menciona una preparación previa de la muestra	Lavado con agua y secar con Na2SO4 anhidro
Preparación de 4 soluciones de calibración utilizando como solvente el blanco de glicerina y como soluto metanol de alta pureza	Se emplean 3 soluciones de calibración, usando de referencia FAME y agregando metanol puro	Se emplean 3 soluciones de calibración, usando de referencia FAME y agregando metanol puro	Se emplean 6 soluciones de calibración con sus replicas
4 puntos de concentración en porcentaje másico de los patrones de la curva de calibración son: 0,125%; 0,25%; 0,50% y 1,00%	La concentraciones obtenidas de los patrones fueron de 0.5, 0.1 y 0.01% m/m	La concentraciones obtenidas de los patrones fueron de 0.5, 0.1 y 0.01% m/m	Las concentraciones de los puntos de calibración fueron 0.0057, 0.011, 0.023, 0.034, 0.045, 0.057, 0.11, 0.23, 0.57, 1.14, 1.70, 2.27 % m/m
Volumen de inyección de la fase de vapor es un 1mL	2 ml de cada solución se transfirieron a viales de espacio de cabeza	1mL de muestra	1mL de muestra
Las condiciones de temperatura del horno cromatográfico durante el análisis 70°C por 7 minutos, 20°C/min hasta 150°C por tres minutos	Programa en el horno: 50°C (1min) a 130°C, 10°C/min, mantener por 0,5min	Horno: 80°C (0,5min isotérmico), 20°C/min hasta 160°C (2min)	Programa horno: temperatura inicial de 40°C por 4min, incrementa a 120°C a 10°C/min y se incrementa a 200°C a 20°C/min, se mantiene a 200°C por 1min
La temperatura del inyector 200°C y la temperatura del detector 250°C	Temperatura del detector 250°C y del inyector 160°C	La temperatura del inyector 250°C y la temperatura del detector 275°C	La temperatura del inyector 200°C y la temperatura del detector 300°C
Empleo de columna de polietilenglicol de 30m*0,32mm*0,5µm	Columna no polar de 30m, 0.32mm ID, 3.0 µm f.t.	Varian Select™ Biodiesel para metanol, 30m*0.32mm*3.0m, CP-9083	Columna HP-5 (5%-fenil)-metilpolisiloxano
Relación Split 50:1	No se menciona	Relación Split 50:1	Relación Split 20:1
Cuantificación de glicerina que tengan un contenido de 0,125% a 1,00% de metanol	Un contenido de metanol de 0.01% m/m a 0.5% m/m	Un contenido de metanol de 0.01% m/m a 0.5% m/m	Se logro detectar un 0.25% de metanol

Los documentos del estado de la técnica cercanos a la invención corresponden a D2, D3 y D4 porque:

El documento D2 enseña un método de determinación del contenido de metanol en biodiesel puro adoptando los métodos estándar que caracterizan un biodiesel,

el método empleado se utiliza un Thermo Scientific TRACE GC Ultra equipado con un split/splitless (SSL) de entrada y un detector de ionización de llama (FID), automatizado con un inyector automático TriPlus, configurado para utilizar el espacio de cabeza. La columna analítica era no polar Thermo Scientific TRACE TR-BIODIESEL (M), 30m, 0.32mm de diámetro, 3.0µm. para la calibración se empleo metanol con una pureza >99.5% y una referencia de esteres metílicos de de ácidos grasos (FAME) con un contenido de metanol <0.001% m/m. en la preparación de la muestra, la muestra de referencia FAME se preparara con una extracción con agua asegurando el contenido bajo en metanol (0.001% m/m). Se emplean 3 soluciones de calibración, usando de referencia FAME y agregando metanol puro, donde las concentraciones obtenidas de los patrones fueron de 0.5, 0.1 y 0.01% m/m, luego 2 ml de cada solución se transfirieron a viales de espacio de cabeza, los parámetros obtenidos fueron, para el programa en el horno del GC empleado: 50°C (1min) a 130°C, 10°C/min, mantener por 0,5min, la temperatura del detector 250°C y del inyector 160°C, todo para determinar un contenido de metanol de 0.01% m/m a 0.5% m/m (folios 52 – 55)

El documento D3 enseña un método de determinación del contenido de metanol en biodiesel usando cromatografía de gases con espacio de cabeza de acuerdo a la norma EN-14110, donde se empleo un equipo Varian 430-GC: -inyector: Split / splitless 1177, -detector: FID, muestreador de espacio de cabeza -QUMA, QHSS-40 modo muestra loop; como columna una Varian Select TM Biodiesel para metanol, 30m×0.32mm×3.0µm, CP-9083. Se emplearon como soluciones de calibración: -solución A: 0.5% (m/m) metanol en FAME, -solución B: 0.1% (m/m) metanol en FAME, -solución C: 0.01%(m/m) metanol en FAME, luego una alícuota de 1 ml se pesaron con exactitud, y se transfirió a un vial de 20 ml y se tapó inmediatamente. Las condiciones del cromatógrafo de gases: -inyector: 250°C, velocidad Split: 50:1, detector: 275°C, FID, horno: 80°C (0,5min isotérmico), 20°C/min hasta 160°C (2min), gas de corrida: flujo constante de helio 2.0mL/min (folios 56 y 57).

El documento D4 enseña un estudio en la determinación de metanol en biodiesel por microextracción en fase solida headspace, donde se mencionan ciertas características de la microextracción en fase solida, tales como sus ventajas, las cuales incluyen que no se requiere solvente, que es simple y rápida y que hay dos modos posibles de extracción del analito, espacio de cabeza e inmersión directa. Al igual se menciona las desventajas tales como el limitado tiempo de vida media de la fibra, la cual se degrada con el uso. En cuanto al método de análisis se menciona que, para a la muestra de biodiesel se le realiza un lavado con agua y se seca toda la noche con Na₂SO₄ anhidro. En la calibración se emplean 6 soluciones de con sus respectivas replicas, donde las concentraciones fueron 0.0057, 0.011, 0.023, 0.034, 0.045, 0.057, 0.11, 0.23, 0.57, 1.14, 1.70, 2.27 % m/m (metanol/biodiesel), se emplea un 1mL de muestra y el programa en el horno fue: temperatura inicial de 40°C por 4min, incrementa a 120°C a 10°C/min y se incrementa a 200°C a 20°C/min, se mantiene a 200°C por 1min, la temperatura del inyector 200°C y la temperatura del detector 300°C, se emplea una columna HP-5 (5%-fenil)-metilpolisiloxano, una relación Split 20:1; finalmente se logra detectar un 0.25% de metanol (folios 58 – 61, paginas 5901 – 5905).

La diferencia entre la invención, definida en las reivindicaciones 2 – 10, y el método de D2 consiste en las condiciones cromatográficas, tales como condiciones en el horno, columna empleada, entre otras.

La diferencia entre la invención, definida en las reivindicaciones 2 – 10, y el método de D3 consiste en las condiciones cromatográficas, tales como condiciones en el horno, columna empleada, entre otras, además que en D3 no se menciona la preparación previa de la muestra.

La diferencia entre la invención, definida en las reivindicaciones 2 – 10, y el método de D4 consiste en las condiciones cromatográficas y el hecho de que se empleó una microextracción en fase solida.

No existe evidencia que las diferencias antes mencionadas entre el método de D2 o el de D3 o el de D4 y el método definido en las reivindicaciones 2 – 10 introduzcan un efecto técnico inesperado, que represente para la persona de nivel medio versada en la materia una mejor eficiencia en la determinación de metanol.

Además como se observó ya es bien conocido en el estado de la técnica que existen varias adaptaciones de la norma EN-14110 para determinar el contenido de etanol y directamente en el biodiesel por cromatografía de gases con sistema headspace. Al igual la técnica de preparación de la muestra es ampliamente conocida² e incluso ha sido modificada a la microextracción en fase solida la cual ha sido ampliamente utilizada en concentraciones de los ppm e incluso los ppt, además se ha demostrado una versatilidad gratamente sorprendente y ha sido empleado para una enorme variedad de problemas analíticos y se menciona el procedimiento de espacio de cabeza-SPME que incluye la extracción, seguida de la desorción en la entrada clásica de un GC splitless³, e incluso este sistema se emplea para determinar metanol en otras matrices, por ejemplo en la pared de un tipo de planta⁴.

Por lo tanto, el problema técnico objetivo a resolver es plantear una alternativa a los métodos conocidos según D2 o D3 o D4 para mejorar la eficiencia en la determinación de metanol.

En consecuencia, el experto en la materia, teniendo en cuenta las enseñanzas mencionadas y los métodos de D2 o D3 o D4 para determinar el contenido de metanol para llegar así al objeto de las reivindicaciones 8 – 10 de la solicitud. Por lo cual se considera obvia.

Conclusión:
Las reivindicaciones 8 – 10 cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

6. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	PerkinElmer, ecoanalytix	1	Nivel Inventivo
D2	Thermo Scientific Mumari F. et Al	1 – 10	Nivel Inventivo
D3	Agilent Technologies, Application Note SI-0773, Coen Duvekot, Varian, Inc.	1 – 10	Nivel Inventivo
D4	Maria C, et Al, Bioresource Technology, vol 99, pages 5901 - 5905	2 – 10	Nivel Inventivo

¹ Modern Practice of gas Chromatography (2004), Wiley Interscience, Estados Unidos, cap 17 (folios 45 – 47)

² Harris D. (2003). Análisis químico cuantitativo. Barcelona – España, pp 596 – 601 (folios 38 – 44).

³ Snow N., (2002). Head-Space analysis in modern gas chromatography. *Trends in analytical chemistry*, vol. 21, pp 608 – 616 (folios 62 – 71).

⁴Nunes C. et Al (2006). Simple and solvent – free methodology for simultaneous quantification of methanol and acetic acid content of plant polysaccharides based on headspace solid phase microextraction-gas chromatography (HS-SPME-GC-FID). *Carbohydrate Polymers*, 64, pp 306 – 311 (folios 72 – 77).

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante y presentarla por escrito

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.


JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha
09 OCT. 2012

Elaborado por: Mayra Alejandra Quintero
Revisado por: Gloria Jacqueline Alfonso

DANIEL C. HARRIS

Análisis químico cuantitativo

3ª Edición *(Sexta edición original)*

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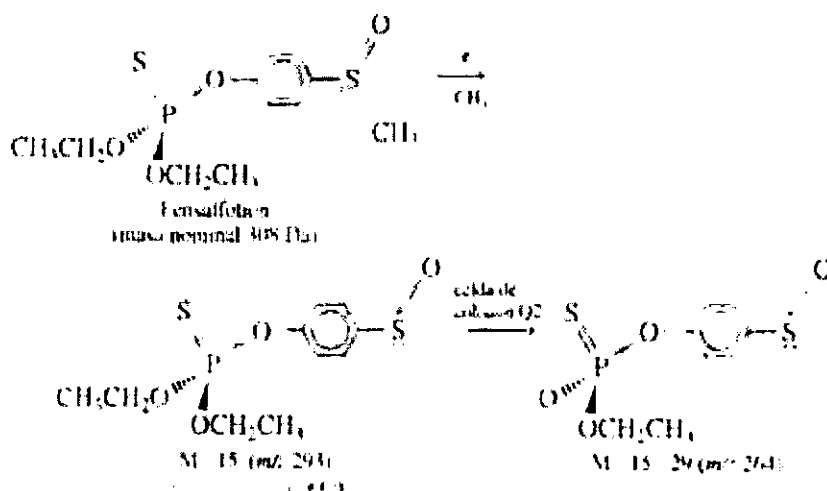
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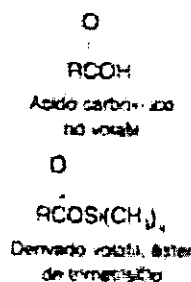
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La detección de reacciones seleccionadas aumenta la relación señal/ruido en los análisis cromatográficos y elimina casi todas las interferencias.



Ejemplo de derivatización:



24.4 Preparación de muestra

La preparación de muestra es el proceso de transformación de una muestra en una forma adecuada para ser analizada. Este proceso puede incluir la extracción de un analito de una matriz compleja, la *preconcentración* de analitos muy diluidos, hasta obtener una concentración suficiente para ser medida, la eliminación o enmascaramiento de especies interferentes, o la transformación del analito en una forma que pueda ser más fácilmente detectable. Un ejemplo de transformación química de analito, también llamada *derivatización*, si muestra en el recuadro 24.1, donde se explica la conversión de aminoácidos no volátiles en derivados volátiles, que se pueden determinar por cromatografía de gases. El capítulo 28 se dedica a la preparación de muestras, de modo que aquí sólo se comentan dos técnicas que son aplicables especialmente en cromatografía de gases.

La microextracción en fase sólida es un método sencillo de extracción de líquidos aire, o incluso todos, sin usar disolventes.¹¹ El componente clave es una fibra de sílice fundida recubierta de una fina película, de 10 a 100 µm, de un líquido no volátil, semejante a las fases estacionarias utilizadas en cromatografía de gases. La figura 24.19 muestra una de estas fibras incorporada a la base de una jeringa con un agujero metálico fijo. La fibra puede emerger de la aguja y se puede introducir dentro de ella. La fibra puede ser utilizada para la extracción de un líquido durante un tiempo, mientras se agita, y si conviene también calentando. Lo mejor es determinar experimentalmente cuánto tiempo se necesita para que la fibra se sature con el analito, y prolongar la extracción durante ese tiempo. Si se acorta el tiempo de extracción, probablemente la concentración de analito en la fibra variará de muestra a muestra. Aun cuando se alcance el equilibrio, en la fibra sólo se extrae una fracción del analito que hay en la muestra. Cuando se extrae del espacio de cabeza, la muestra líquida debe ocupar > 2/3 del vial. Demasiado volumen de espacio de cabeza reduce la eficiencia de la extracción.

Una vez tomada la muestra, se repliega la fibra y se introduce la aguja dentro de un cromatógrafo de gases. Se saca la fibra dentro de la guía caliente del inyector, en cuyo interior el analito se desorbe térmicamente de la fibra, y se introduce sin división durante un tiempo fijo. Una concentración por frío (apartado 24.2) concentra (localiza) el analito desorbido en la cabeza de la columna antes de hacer la cromatografía. Si pasa mucho tiempo entre la toma de muestra y la inyección, se debe pinchar la aguja en un septo, para mantener la muestra perfectamente sellada. La figura 24.21 muestra un cromatograma de productos de guerra química, que actúan sobre el sistema nervioso, aislados de agua de mar por microextracción en fase sólida, y detectados por el sistema nervioso. El cromatograma puede parecer demasiado simple, pero eso es debido a que el detector sólo responde a compuestos que contienen N y P.

En una microextracción, la cantidad de analito (m , µg) absorbido en una fibra recubierta vale

$$\text{Masa de analito extraído} \quad m = \frac{KV_f C_0 V_0}{KV_f + V_0} \quad (24.10)$$

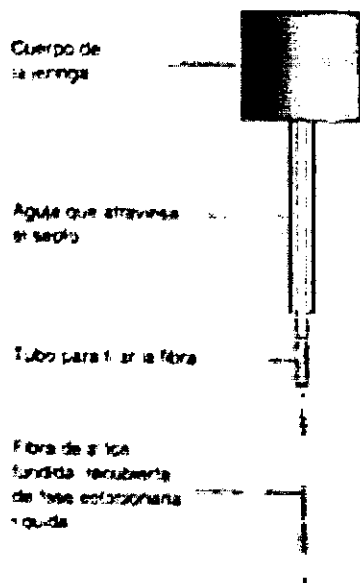


Figura 24.19 Jeringa para microextracción en fase sólida. La fibra de sílice fundida se repliega dentro de la aguja de acero, después de haber recogido la muestra, y cuando se viene que pinchar la jeringa en un septo.

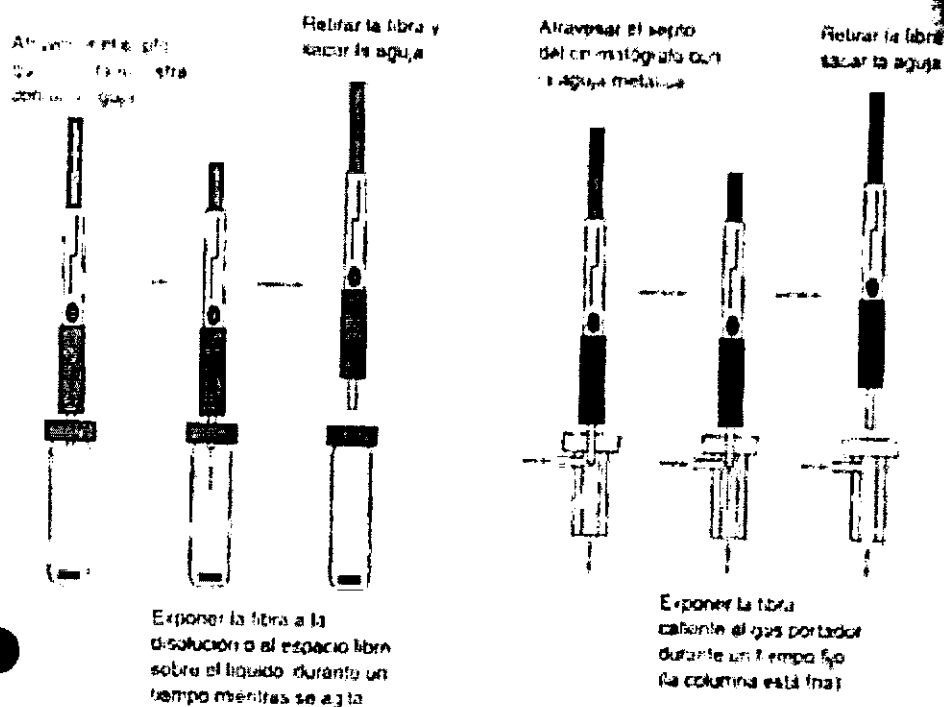


Figura 24.20 Toma de muestra mediante microextracción en fase sólida y desorción de analito de la fibra recubierta, para su introducción en un cromatógrafo de gases. (Tomado de *Supercritical Chromatography*, Product Catalog, Belleronte PA.)

donde V_f es el volumen de la película de la fibra, V_d es el volumen de la disolución de la que se extrae, y C_0 es la concentración inicial (ppg/mL) de la disolución que se extrae. K es el coeficiente de reparto del soluto entre la película y la disolución. $K = C_f/C_d$ donde C_f es la concentración del soluto en la película y C_d la concentración en la disolución. Si se extrae un volumen grande de disolución, tal que $V_d \gg KV_f$, la ecuación se reduce a $m = KV_f C_0$. Es decir, la masa extraída es proporcional a la concentración del analito en la disolución. Para hacer un análisis cuantitativo se construye una curva de calibrado haciendo extracciones de disoluciones conocidas. En microextracción en fase sólida también son útiles el patrón interno y la adición de patrón.

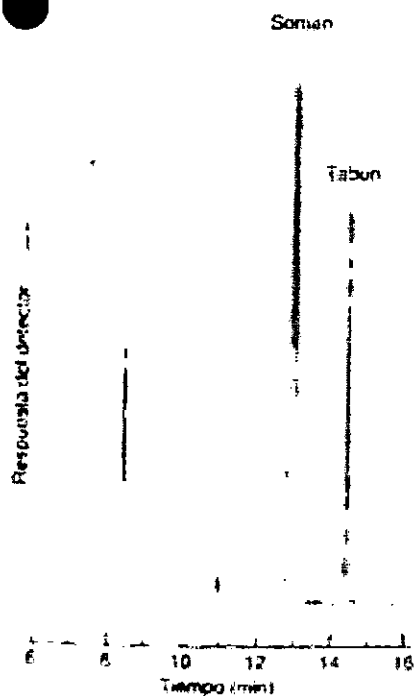
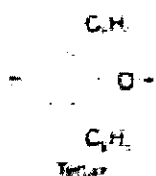


Figura 24.21 Cromatograma de gases de productos de guerra química aislados de agua de mar por microextracción en fase sólida durante 30 min, a la que se ha añadido 60 nL de cada producto por litro de agua (60 ppb en volumen). La fibra tiene un recubrimiento de copoli(dimetilsiloxano-divinilbenceno) de 65 μm de espesor. El detector de nitrógeno-fósforo tiene un límite de detección de 0.05 ppb. Los analitos se desorbieron de la fibra durante 2 min a 250 $^{\circ}\text{C}$, y se inyectaron en el cromatógrafo sin división. La temperatura de la columna era de 30 $^{\circ}\text{C}$ durante la desorción, y después fue subiendo a razón de 10 $^{\circ}\text{C}/\text{min}$ durante la cromatografía. La columna tenía 0.32 mm de diámetro y 30 m de longitud, y un recubrimiento de $(\text{dimethylsiloxane})_{95}/(\text{methylstyrene})_{5}$ por oxígeno de 1 μm de espesor. El soman tiene un pico desdoblado en dos, porque tiene dos isómeros. (Tomado de H. A. Lammey y W. F. Hays, "Determination of Chemical Warfare Agents in Natural Water Samples by Solid-Phase Microextraction," *Anal. Chem.* 1997, 69, 1865.)



Se necesita determinar el tiempo y la temperatura necesario para purgar el 100% del analito de la muestra mediante varios ensayos independientes de control.

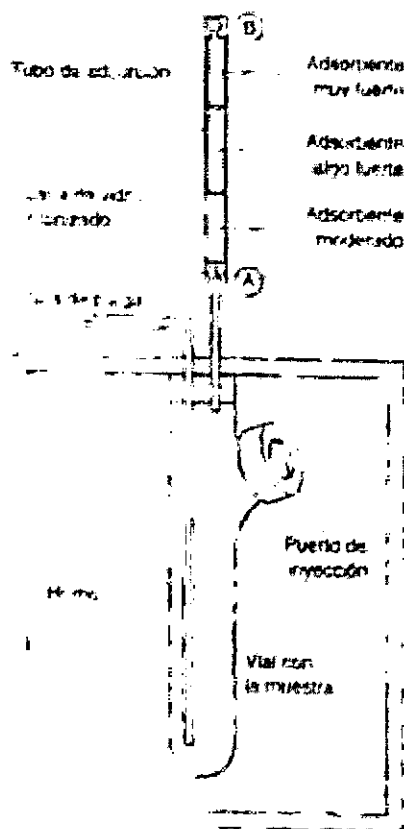


Figura 24.22 Aparato de purga y trampa para extraer sustancias volátiles de un líquido o sólido mediante una corriente de gas.

El método de purga y trampa sirve para separar analitos volátiles de líquido o sólidos como agua, sustancias o suelos, concentrarlos e introducirlos en un cromatógrafo de gases. A diferencia de la microextracción, que sólo aísla una porción del analito que hay en la muestra, el objetivo de este método es aislar el 100% del analito de la muestra. La separación total de analitos polares de matrices polares puede ser muy difícil.

La figura 24.22 muestra un aparato para medir componentes volátiles del aroma de bebidas carbonicas de cola. Para ello se hace burbujear He , como gas de purga, mediante una aguja de acero inoxidable a través de la cola, colocada en el vial de muestras, calentado a 50°C para ayudar a la evaporación de los analitos. El gas de purga que sale del vial pasa por un tubo de adsorción, que contiene tres capas de compuestos adsorbentes, de poder adsorbente cada vez mayor. Por ejemplo, el adsorbente moderado podría ser uno no polar, tenilmetilpolisiloxano, el adsorbente algo más fuerte, el polímero Tenax, y el adsorbente más fuerte, tamices moleculares de carbono.

Durante el proceso de purga y trampa de la figura 24.22, el gas fluye por el tubo adsorbente desde el extremo A al extremo B. Después de atravesar todo el analito de la muestra hasta el tubo de adsorción, se invierte el sentido del flujo, haciéndolo ir de B a A, y se purga la trampa a 25°C para eliminar toda el agua y cualquier otro disolvente que puedan tener los adsorbentes. La salida A del tubo de adsorción se conecta entonces con el inyector de un cromatógrafo de gases, funcionando sin división, y la trampa se calienta a unos 200°C . Los analitos desorbidos penetran en la columna del cromatógrafo, donde se concentran en una trampa fría. Una vez se ha completado la desorción de la trampa, se calienta la columna cromatográfica para iniciar la separación.

La nave espacial Cassini, que se lanzó en 1997, llegó a Saturno en 2004. La sonda Huygens se separó de la nave Cassini, y descendió en paracaídas a través de la atmósfera de la luna de Saturno, Titan. Esta sonda tenía dos trampas de carbón adsorbente. Una trampa adsorbía los hidrocarburos desde C_2 hasta C_8 , y la otra adsorbía gases «permanentes» como H_2 , He , CH_4 , NH_3 y gases nobles. Los gases se desorbieron térmicamente y se analizaron en la nave mediante cromatografía de gases/espectrometría de masas.

24.5 Puesta a punto de un método en cromatografía de gases

Dada la cantidad de parámetros que hay que fijar en cromatografía de gases, ¿existe un procedimiento lógico para seleccionar un procedimiento para resolver un problema concreto? En general, existen muchas soluciones satisfactorias. En este apartado se comentan normas generales para seleccionar el método que se tiene que usar.⁽¹⁾ El orden como se deben tomar las decisiones es considerar: (1) lo que se pretende en el análisis, (2) la preparación de muestra, (3) el detector, (4) la columna y (5) la inyección.

Objetivo del análisis

¿Qué se busca con el análisis? ¿Es la identificación cualitativa de los componentes de una mezcla? ¿Se precisa una separación de gran resolución entre todos los componentes, o simplemente de una sola porción del cromatograma? ¿Se puede sacrificar la resolución para ganar en rapidez? ¿Se necesita un análisis cuantitativo de uno o de muchos componentes? ¿Se necesita una gran precisión? ¿Se encuentran los analitos en concentración suficiente o se necesitan técnicas especiales (preconcentración y un detector muy sensible) para análisis de ultratrazas? Cada uno de estos factores conduce a ciertos compromisos a la hora de seleccionar técnicas.

Preparación de muestra

La clave del éxito para analizar por cromatografía una muestra compleja es su purificación antes de introducirla en la columna. En el apartado anterior se describieron la microextracción y la purga y trampa como dos métodos para aislar componentes volátiles de matrices complejas. Otros métodos son la extracción líquida, la extracción con fluidos supercríticos, la extracción en fase sólida y la desorción térmica de materiales sólidos, la mayoría de los cuales se describen en el capítulo 28. Estas técnicas aíslan los analitos de interés de las sustancias interferentes, y pueden concentrar analitos hasta valores detectables. Si no se purifican las muestras, los cromatogramas podrían estar constituidos por «bosques» de picos

sin resolver, y las sustancias no volátiles inutilizarían la columna cromatográfica, que es cara.

Selección del detector

El paso siguiente es elegir un detector para la cromatografía. ¿Se necesita información sobre todo lo que hay en la muestra, o se necesita un detector específico para un elemento o un grupo de elementos en particular?

El detector de aplicación más general para una cromatografía con columna tubular abierta es un espectrómetro de masas. La ionización de llama es probablemente el detector más popular, pero responde sobre todo a hidrocarburos, y tal como muestra la tabla 24.5, no es tan sensible como el detector de captura electrónica, el de nitrógeno-fósforo, o los de quimioluminiscencia. El detector de ionización de llama exige que la muestra contenga ≥ 10 ppm de cada analito, si la inyección se hace con división. El detector de conductividad térmica es la forma más general de detectar toda clase de compuestos, pero no es bastante sensible en columnas tubulares de pequeño calibre de alta resolución.

Los detectores sensibles para análisis de ultratrazas responden a una clase limitada de analitos. El detector de captura electrónica es específico de moléculas que contienen halógenos, carbonilos conjugados, nitrilos y nitrocompuestos. Cuando se usa inyección con división, la muestra debe contener ≥ 100 ppb de cada analito, si se usa un detector de captura electrónica. El detector de fotoionización puede ser específico de compuestos aromáticos y compuestos no saturados. El detector de nitrógeno-fósforo da mayor respuesta a compuestos que contienen alguno de estos elementos, pero también responde a los hidrocarburos. Los detectores de quimioluminiscencia de azufre y nitrógeno responden solo a estos elementos. Los detectores de fotometría de llama son específicos para algunos elementos, como S, P, Pb o Sn. Se puede elegir alguno de estos detectores específicos para simplificar el cromatograma, porque no responden a todo lo que se eluye.

Si se necesita información cualitativa sobre la identidad de los eluatos, una buena elección son los espectrómetros de masas o el de infrarrojos. El detector de infrarrojos, al igual que el detector de conductividad térmica, no es bastante sensible para trabajar con columnas tubulares de pequeño calibre de gran resolución.

Selección de columna

Las elecciones básicas son de fase estacionaria, de diámetro y longitud de columna, y de espesor de fase estacionaria. La fase estacionaria más útil es una no polar, como alguna de la tabla 24.1. Una fase estacionaria de polaridad intermedia podrá llevar a cabo más separaciones que una columna no polar. Para compuestos muy polares puede ser necesaria una fase estacionaria muy polar. La separación de isómeros ópticos o isómeros geométricos muy semejantes precisan fases estacionarias especiales.

La tabla 24.6 muestra que existen pocas combinaciones sensatas de diámetro de columna y espesor de película. Para lograr la máxima resolución se precisan columnas estrechas, con espesores de fase estacionaria muy finos. Esta combinación minimiza la resistencia a la transferencia de masa (el término C de la ecuación de van Deemter, ecuación 23.33), tanto en la fase móvil como en la estacionaria, reduciendo así la altura de plato. Película fina y columnas estrechas son especialmente adecuadas para separar mezclas de compuestos de alto punto de ebullición, que se retienen demasiado en columnas de película gruesa. Los tiempos de retención breves permiten análisis muy rápidos. Sin embargo, las columnas estrechas de películas finas tienen una capacidad de muestra muy pequeña, necesitan detectores de gran sensibilidad (el de ionización de llama puede que no sea adecuado), no retienen bien los compuestos de punto de ebullición bajo, y podrían verse afectadas por la exposición de los sitios activos de la sílice.

Las columnas estrechas de película gruesa de la tabla 24.6 constituyen un buen compromiso entre resolución y capacidad de muestra. Pueden usarse con la mayoría de los detectores (excepto, en general, de los de conductividad térmica o de infrarrojos) y con compuestos muy volátiles. Los tiempos de retención son mayores que los de columnas de película fina. Cuando se usan detectores de conductividad térmica o de infrarrojos se precisan columnas anchas de película gruesa. Estas tienen gran capacidad de muestra, y pueden separar compuestos muy volátiles, pero dan baja resolución y tienen tiempos de retención grandes.

Orden decisiones

- finalidad del análisis
- preparación de muestra
- detector
- columna
- inyección

Tabla 24.6

Descripción	Estrechitas de película fina	Estrechitas de película gruesa	Anchas de película gruesa
Diámetro interior	0,10-0,32 mm	0,25-0,32 mm	0,53 mm
Espesor de película	~0,2 µm	1-2 µm	~2-5 µm
Ventajas	Resolución alta Análisis de trazas Separaciones rápidas Bajas temperaturas Fluyen compuestos de punto de ebullición alto	Buena capacidad Buena resolución (4 000 platos/m) Fácil de usar Retiene compuestos volátiles Buenas para espectrometría de masas	Capacidad alta (100 ng/soluto) Buenas para detectores de conductividad e intrínsecos Técnicas sencillas de inyección
Desventajas	Capacidad baja (≤1 ng por soluto) Requiere detectores de gran sensibilidad (no, espectrometría de masas) Actividad superficial de la sílice expuesta	Resolución moderada Tiempos de retención elevados en compuestos de alto punto de ebullición	Resolución baja (500-2 000 platos/m) Tiempos de retención elevados en compuestos de alto punto de ebullición

Para mejorar la resolución, usar
una columna más larga
una columna más estrecha
una fase estacionaria más fina
otra fase estacionaria

Si una columna cumple la mayoría de los objetivos, pero no tiene suficiente resolución, se puede usar una columna más larga del mismo tipo. Duplicando la longitud de columna, se duplica el número de platos, y según la ecuación 23.30, la resolución aumenta en un factor de $\sqrt{2}$. No es éste necesariamente el mejor modo de aumentar la resolución, porque duplica el tiempo de retención. Usando una columna más estrecha o película de fase estacionaria más fina la resolución aumenta sin la penalización de aumento de tiempo de retención. Si se cambia la fase estacionaria, cambia completamente la retención relativa de los diferentes compuestos (n en la ecuación 23.30), y puede ser que así se resuelvan los compuestos que interesa separar.

Si se ha medido la resolución de unos pocos componentes clave de una mezcla variando un pequeño número de condiciones, se puede utilizar un software comercial para optimizar las condiciones de una mejor separación (como la programación de temperatura y de presión).¹⁶ En un futuro próximo se prevé que se puedan conseguir separaciones mucho mejores acoplando dos columnas diferentes en serie, con un control programado de caudales (presión) en cada una de ellas durante la separación.¹⁷

Elección del modo de inyección

La última decisión importante es cómo inyectar la muestra. Cuando la concentración de analito es alta o se analizan gases, el mejor modo de hacerlo es por *inyección con división*. Para análisis cuantitativo resulta muy pobre. Durante la inyección se pueden perder compuestos volátiles. La inyección con división da una gran resolución y puede tratar muestras complejas, si se añade un lecho de adsorbente en la guía del inyector. Los compuestos térmicamente inestables se pueden descomponer durante la inyección a temperatura alta.

Se precisa una *inyección sin división* cuando las disoluciones son muy diluidas. Da gran resolución, pero es pobre en análisis cuantitativo, porque se pueden perder compuestos menos volátiles durante la inyección. Es mejor que el modo con división para compuestos de moderada estabilidad térmica, porque la temperatura de inyección es menor. La inyección sin división introduce la muestra lentamente en la columna, de modo que si

Inyección con división

muestra concentrada
resolución alta
muestras fáciles (usar guías con adsorbente)
puede provocar descomposición térmica

Inyección sin división

muestra diluida
resolución alta
precisa rampa de disolvente o trampa fría

requiere una trampa de disolvente o una trampa fría. Por tanto, la inyección sin división no se puede usar en cromatografía isoterma. Las muestras que contienen menos de 100 ppm de cada analito se pueden analizar con columnas de un grosor de película de $< 1 \mu\text{m}$ con inyección sin división. Las muestras que contienen entre 100 y 1000 ppm de cada analito requieren columnas de $\approx 1 \mu\text{m}$ de grosor de película.

En análisis cuantitativo, y en el caso de compuestos sensibles térmicamente, es mejor la inyección en columna. Estrictamente, es una técnica de resolución baja, y no puede usarse con columnas cuyo diámetro interior sea menor que 0,25 mm. Puede utilizarse con disoluciones diluidas y concentradas, y con volúmenes relativamente grandes o pequeños. Los demás requisitos de columna son los mismos que en inyección sin división.

Inyección en columna:

- óptima para análisis cuantitativo
- compuestos térmicamente sensibles
- resolución baja

Términos importantes

Co-cromatografía	Detección de una reacción seleccionada	Gas portador	Preparación de muestra
Columna empaquetada	Detector de captura electrónica	Índice de retención	Programación de temperatura
Columna tubular abierta	Detector de conductividad térmica	Inyección con división	Purga y trampa
Concentración por frío	Detector de ionización de llama	Inyección en columna	Septo
Condensación de disolvente		Inyección sin división	Tamiz molecular
Cromatografía de gases		Microextracción en fase sólida	
Detección de iones seleccionados			

Resumen

La cromatografía de gases utiliza una fase móvil gaseosa para hacer pasar un líquido volátil o un soluto gaseoso por una fase estacionaria que se encuentra dentro de una columna tubular abierta o sobre un soporte sólido. Las columnas tubulares abiertas largas y estrechas tienen poca capacidad, pero permiten excelentes separaciones. Las columnas tubulares abiertas de sílice fundida pueden ser de pared recubierta, recubiertas de soporte y de capa porosa. Las columnas empaquetadas tienen gran capacidad, pero una resolución limitada. Las fases estacionarias retienen, sobre todo, los solutos de polaridad semejante ("lo semejante disuelve a lo semejante"). Las fases estacionarias sólidas pueden ser de carbón poroso, alúmina y tamices moleculares. El índice de retención mide los tiempos de salida o elución en relación con el de los alcanos. La programación de temperatura y de presión reduce los tiempos de elución de los componentes fuertemente retenidos. Sin comprometer la eficacia de la separación, se puede aumentar el caudal usando H_2 o He , en lugar de N_2 , como gas portador. La inyección con división proporciona separaciones de gran resolución de disoluciones relativamente concentradas. La inyección sin división, que es necesaria en muestras muy diluidas, precisa que se elimine el disolvente, o que se concentren los analitos mediante una trampa fría al principio de la columna (para conseguir bandas estrechas). La inyección en columna es la mejor forma de inyección en análisis cuantitativo, y en el caso de compuestos térmicamente inestables.

El análisis cuantitativo se suele hacer en cromatografía de gases con patrones internos. La co-cromatografía de una muestra auténtica con una desconocida en diferentes columnas es útil para la identificación cualitativa de un pico. Los detectores de espectrometría de masas y de infrarrojos dan información cualitativa, que sirve para identificar compuestos desconocidos. El espectrómetro de masas se ha convertido en el detector más sensible y menos sujeto a interferencias, cuando se emplea detección de iones seleccionados o detección de una reacción seleccionada. La detección por conductividad térmica tiene validez universal, pero no es bastante sensible para columnas tubulares abiertas de diámetro reducido. El detector de ionización de llama es bastante sensible para la mayoría de las columnas, y responde a la mayoría de los compuestos orgánicos. Los detectores de captura electrónica, nitrógeno-flúor, fotometría de llama, fotoionización, quimioluminiscencia y emisión atómica son específicos de ciertas clases de compuestos o de elementos determinados.

Es preciso decidir la finalidad de un análisis antes de diseñar un método analítico. La clave del éxito en cromatografía es tener una muestra «limpia» (lo más pura posible). La microextracción en fase sólida y la técnica de purga y trampa son métodos de preparación de muestras que aíslan los componentes volátiles de muestras complejas. Una vez escogido el método de preparación de muestra, para diseñar un método hay que tomar las siguientes decisiones, por este orden: seleccionar un detector, una columna, y un método de inyección.

Ejercicios

4 A. a) Cuando se analizó por cromatografía de gases una disolución que contenía 234 mg de butanol (MF 74,12) y 312 mg de hexanol (MF 102,17) en 10,0 mL, las áreas relativas de los picos butanol/hexanol fueron = 1,00:1,45. Considerando al butanol como el patrón interno, hallar el factor de respuesta del hexanol.

b) Usar la ecuación 24.2 para estimar las áreas de los picos del butanol y del hexanol en el cromatograma de gases de la página siguiente.

c) La disolución con la que se obtuvo el cromatograma contenía 112 mg de butanol. ¿Cuánto hexanol había en la disolución?

MODERN PRACTICE OF GAS CHROMATOGRAPHY

FOURTH EDITION

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CHAPTER SEVENTEEN

Validation and QA/QC of Gas Chromatographic Methods

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17.1 INTRODUCTION

As with any equipment, the gas chromatograph requires validation before being used in an analytical method. Validation is a critical part of the development of an analytical gas chromatographic procedure. It is what demonstrates accuracy and precision. Validation is defined as "establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its predetermined specifications and quality attributes" (1). It is generally concerned with a specific analysis or method. Validation of a method, however, begins way before samples can be prepared. It begins with the installation and qualification of the instrument and continues with good quality assurance/quality control (QA/QC) in the laboratory.

Quality assurance is a system of activities whose purpose is to prevent problems from occurring and provides assurance that the instrument meets defined standards of quality with a stated level of confidence. The International Organization for Standardization (ISO) provides a general guide for quality assurance programs and analytical laboratories are using these more. ISO Guide 17025 is the standard that many analytical laboratories, including crime laboratories, follow.

While most laboratories realize the need for a good QA/QC program, it is seldom clear which program meets the needs of a particular laboratory, especially if that laboratory is performing a wide variety of analytical services. QA/QC, therefore, can have different meaning to different chromatographers depending on their particular area of interest and standards. However, there are basic similar key elements to a good quality assurance program in gas chromatography that must be addressed. These are designed to ensure that the sample was free from all possible contamination, that it was handled appropriately by trained analysts using calibrated and maintained gas chromatographs and standard validated procedures, and that all of this was appropriately documented. The key elements to a good QA/QC program for gas chromatography will be discussed in greater detail throughout this chapter.

17.2 PROCUREMENT

The first step to ensure quality of the gas chromatograph is the procurement itself. Capabilities and specifications of gas chromatographs vary. Manufacturers

publish technical specifications for their instruments, but those specifications may not be meaningful to your application. They may also include unique, and sometimes useless, characteristics that can be used to preempt another manufacturer from meeting that specification. It is important to procure the correct instrument for your needs. Do you need an instrument with high sensitivity, high precision, or versatility? Is it important to be able to accommodate dirty samples, a variety of matrices, and long runtime between calibrations? What types of injectors and detectors are best for your work? The best way to get the correct instrument is through a performance specification. Determine the realistic performance characteristics that are important to your analyses, and include them as requirements in your procurement document. Examples of performance specifications include the following:

- Sensitivity*—1 pg of heptachlor epoxide must give a response (peak height) greater than 50 times the background noise level.
- Carryover*—the peak area from an injected blank must be less than 0.001% of the peak area of a 100-µg/mL ethanol injection that immediately precedes the blank injection.
- Linearity*—the response, measured as peak area, must be linear (correlation coefficient greater than 0.99995) over three orders of magnitude starting with the minimum detection level (3 times the baseline noise).
- Autosampler precision*—relative standard deviation of peak areas of 20 consecutive injections must be less than 0.5%.
- Calibration drift*—the change peak area of a midrange calibration standard over a 6-h period must not exceed 2.0%.

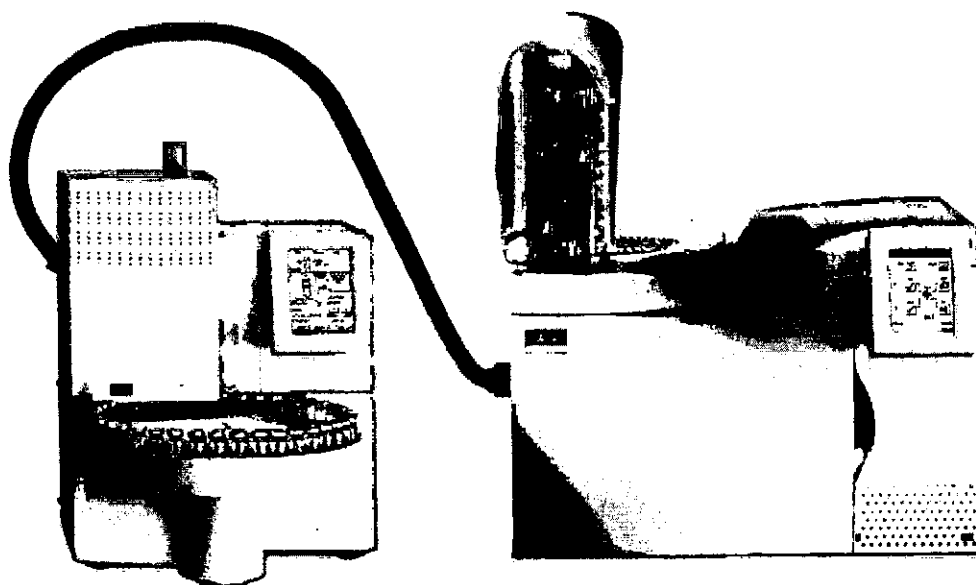
Each instrument performance characteristic that is important to the application should be identified in the procurement specification. The specification will become part of the bid package that is sent to the vendors. In the bid package also state that the vendor must provide evidence with the quotation that the proposed instrument can meet those specifications, and on installation of the instrument, the manufacturer's installer must demonstrate that the instrument installed at your location meets those specifications.

17.2.1 Facilities

The facilities that house gas chromatographic instrumentation are extremely important for producing quality, reproducible data. The design of the laboratory, security, and storage are issues that must be addressed. The laboratory must have a documented security policy that ensures the integrity of the samples by limiting access to areas affecting the quality of testing. Separation of activities that might cause cross-contamination is a prime consideration. Also, control of the environment, especially such variables as temperature and humidity, is extremely important for reproducibility. These are factors that could influence the validity of the test results. Make sure that the temperature and humidity of the laboratory

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Biodiesel Glycerin and Methanol Analyzer



Biodiesel, a renewable fuel produced from natural oil, is used as either a direct substitute for, or an additive to petroleum-based diesel fuel. Interest in biodiesel has increased as a result of rising oil prices and concerns over future supply.

In the production of biodiesel, free fatty acids (lipids) are catalytically converted to fatty acid methyl esters (FAME) with alcohol, typically methanol. Following this reaction, glycerin, water and residual catalyst must be removed to create a fuel suitable for use in compression ignition (diesel) engines. A number of quality problems can arise if the reaction is incomplete or if by-products are not removed effectively.

To ensure fuel quality, both the European Committee for Standardization (EN 14214) and ASTM International (ASTM D6751) have issued standard test criteria for biodiesel. These standards utilize multiple gas chromatographic (GC) analyses.

The PerkinElmer® EcoAnalytix™ Biodiesel Glycerin and Methanol Analyzer provides a unique solution to test biodiesel using the GC test methods included in both the EN and ASTM standards. The analyzer incorporates a TurboMatrix™ Headspace Sampler coupled to a Clarus® GC.

Key Benefits

- ▶ Clarus GC with dual oven allows analysis of both free and total glycerin as well as residual methanol on a single instrument
- ▶ TurboMatrix Headspace Sampler improves productivity and conforms with EN methodology
- ▶ Consumables package with calibration standards provides rapid ramp-up of sampling and analysis
- ▶ Standard operating procedures allow rapid setup of test methods
- ▶ Specific installation and qualification procedures to get the application up and running

The Biodiesel Glycerin and Methanol Analyzer consists of three main components: the TurboMatrix Headspace Sampler, the Clarus GC, and an integrated auxiliary isothermal oven within the GC. The Clarus GC is configured with an autosampler, programmable-on-column injector and two flame ionization detectors. The auxiliary isothermal oven installed within the Clarus GC provides a second temperature-controlled zone for a second chromatographic column. The TurboMatrix Headspace Sampler allows unattended analysis of multiple samples. Figure 1 shows the schematic layout of the 3 components of the system.

The Biodiesel Glycerin and Methanol Analyzer provides the capability to analyze both free and total glycerin (EN 14105, ASTM D6584) as well as residual methanol (EN 14110) with a single, integrated Headspace-GC sampling system.

Figures 2 and 3 (Page 3) demonstrate the chromatography expected when following EN 14105/ASTM D6584 and EN 14110 methodology.

Trouble-free determination of glycerin and residual methanol in biodiesel

The Clarus GC includes programmable pneumatic control (PPC) which allows computerized control of carrier and detector gases, eliminating time-consuming manual interaction. The integral touch-screen user interface provides real-time monitoring and control of the GC.

The independently-controlled second oven allows the analysis of both glycerin and methanol on the Clarus GC.

Table 1. Summary of EN and ASTM methods for biodiesel-quality analysis.

Method	Analytes	Injection	Analysis Time
EN 14105	Free and Total Glycerol, Mono-, Di-, and Triglyceride Content	On-Column	35 min
EN 14110	Residual Methanol	Headspace	< 5 min
ASTM D6584	Free and Total Glycerin	On-Column	25 min

The ability to have two independently controlled ovens reduces the operating cost and optimizes laboratory bench space by combining the methods which formerly required two GCs into a single instrument.

Utilization of programmable on-column injection provides high precision for sample injections. The integral liquid autosampler of the Clarus GC is uniquely suited for on-column injections with superior precision.

Use of metal capillary columns in the Analyzer eliminates many of the handling challenges associated with fused-silica capillary columns and improves the reliability of the system.

The EN 14110 method recommends a 45-minute equilibration time for each sample. The TurboMatrix HS-40 and HS-110 Headspace Samplers thermostat up to 12 samples at a time, ensuring the next sample is ready on completion of the previous run, thereby enhancing throughput to meet the EN method's timing specification.

The GC and Headspace instrumentation require calibration with analytical reference-standard compounds. The Biodiesel Glycerin and Methanol Analyzer includes reference-standard compounds for EN 14105, ASTM D6584 and EN 14110 methods. These reference compounds will allow quick and easy system calibration so that the user can be up and running rapidly and focus on validating the quality of the biodiesel. The reference-standard compounds are also available as individual replacement parts which can be reordered to continue operation of the system.

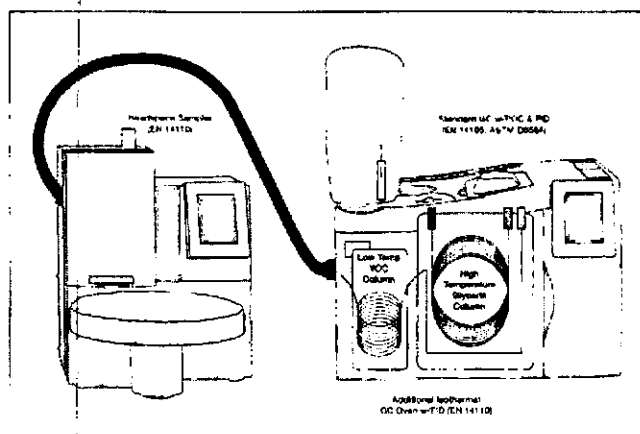


Figure 1. Schematic diagram of the EcoAnalytix Biodiesel Glycerin and Methanol Analyzer, highlighting its unique features.

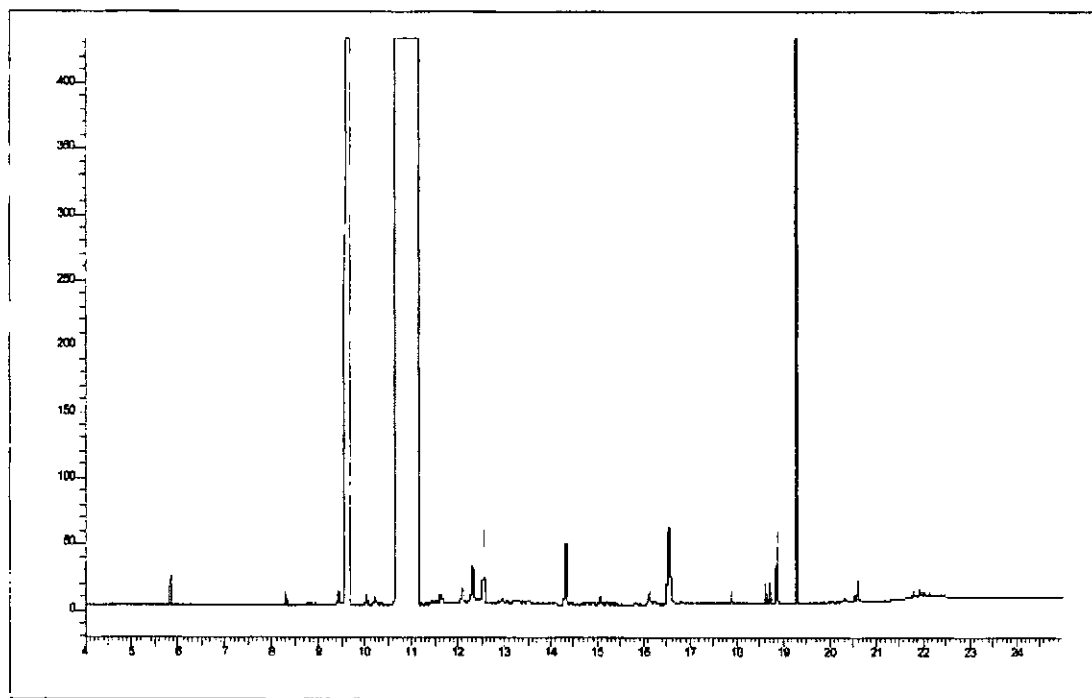


Figure 2. Chromatography demonstrating the analysis of a biodiesel sample for glycerin and mono-, di-, tri-glyceride content.

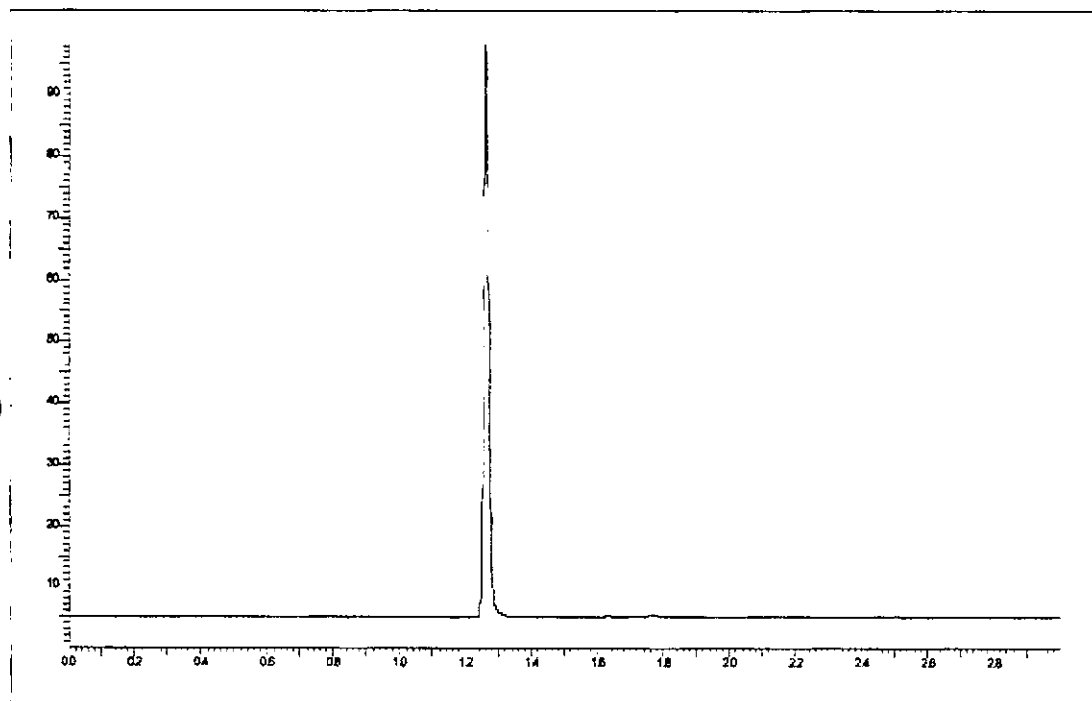


Figure 3. Chromatography demonstrating 0.1% methanol in a biodiesel matrix.

Also included with the Biodiesel Glycerin and Methanol Analyzer is an application CD which includes:

- Application notes with background information on biodiesel GC techniques
- Reference data files for chromatograms
- Software methods to control the GC and Headspace instruments
- Processing methods to translate chromatograms into biodiesel-quality information
- Standard operating procedures (SOPs) for sample preparation, calibration, analysis and reporting

PerkinElmer's global service organization completes the process by providing installation and support. This includes qualification that the Biodiesel Glycerin and Methanol Analyzer meets the needs of the application.

The perfect solution

Complete integration of the Clarus GC with a dual oven, TurboMatrix Headspace Sampler, calibration standards, operating procedures and an application CD makes the PerkinElmer EcoAnalytix Biodiesel Glycerin and Methanol Analyzer the perfect solution to meet the needs of EN 14105, ASTM D6584 and EN 14110 methods in a cost-effective and time-efficient manner.

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Determination of Methanol Content in Pure Biodiesel (B100) by Headspace-GC in Compliance with EN 14110

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Key Words

• TRACE GC Ultra

• Biodiesel (B100)

• EN 14110

• Headspace

• Methanol

Introduction

In order for biodiesel to be commercialized as pure biofuel or blending stock for heating and diesel fuels, it must meet a set of requirements defined in ASTM D6751 and EN 14214 standard specifications.^{1,2} These standards indicate the maximum allowable concentrations of contaminants in pure (B100) finished product, along with other chemical-physical properties necessary for a safe and satisfactory engine operation.

Gas chromatography (GC) is commonly adopted to characterize pure Biodiesel (B100) according to the following standard methods:

- EN 14103: Determination of total FAMES (Fatty Acid Methyl Esters) and Linolenic Methyl Ester (C18:3)^{3,4}
- EN 14105/ASTM D6584: Determination of Free and Total Glycerine^{5,6,7,8}
- EN 14110: Determination of residual methanol content⁹

Comprehensive Thermo Scientific GC solutions have been developed in compliance with each of these methods, based on the Thermo Scientific TRACE GC Ultra™ and the versatile TriPlus™ autosampler (Figure 1). This application note relates to the determination of methanol in biodiesel according to EN 14110.

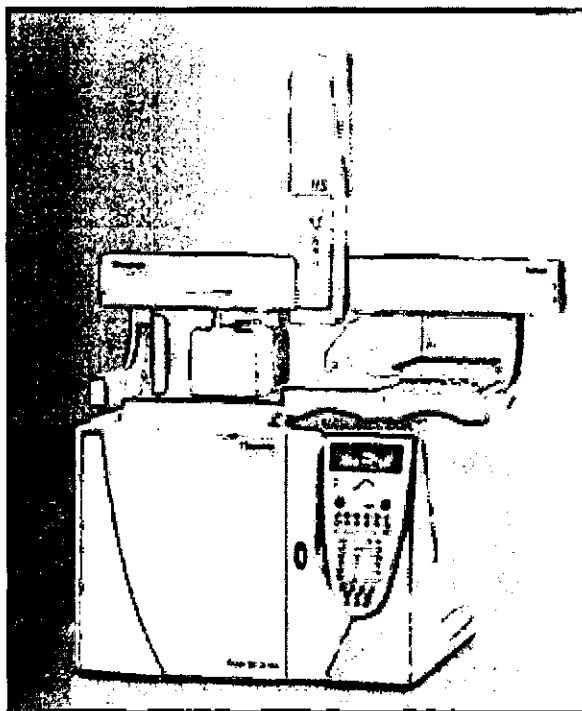


Figure 1: TRACE GC Ultra with TriPlus HS autosampler

Monitoring residual methanol in B100 is a matter of safety since even small amounts of this material can reduce the flash point of the biodiesel. Moreover, residual methanol can affect fuel pumps, seals and elastomers and can result in poor combustion properties. EN 14110 requires a headspace GC method, based on either polar or non-polar columns, and is applicable for a concentration range from 0.01% m/m to 0.5% m/m of methanol (MeOH). The method is not applicable to mixtures of FAME that contain other low boiling components, although the use of the Thermo Scientific TRACE™ TR-BIODIESEL(M) column allows for good separation of methanol from most of these volatiles, producing reliable results.

EN 14214:2003 standard specifications require the methanol content to be < 0.2% m/m. In this note, the analysis was performed using an external calibration procedure, which is preferred over internal standard calibrations when automatic headspace equipment is used.

Methods

Instrumentation and Reagents

A Thermo Scientific TRACE GC Ultra equipped with a split/splitless (SSL) inlet and a flame ionization detector (FID), automated by a TriPlus Autosampler configured for headspace was used, with instrument control through the Thermo Scientific Chrom-Card data system. The analytical column was a non-polar Thermo Scientific TRACE TR-BIODIESEL(M), 30 m, 0.32 mm ID, 3.0 µm f.t. For calibration, methanol with purity > 99.5% and a reference FAME with methanol content < 0.001% m/m were used.

Sample Preparation

A reference sample of FAME can be prepared by extraction with water to ensure low methanol content, taking 30 mL of biodiesel and extracting four times with 10 mL of water. Then, the extracted biodiesel layer must be dried with MgSO₄ for 15 minutes, and analyzed using the parameters reported below. This prepared sample has to provide methanol content less than 0.001% m/m. Such a reference FAME can also be obtained from various commercial sources. In this note, raw sunflower oil has been used, featuring a very low methanol content.

Using the reference FAME, three calibration solutions (A, B and C) were prepared by adding pure methanol to obtain concentrations of 0.5, 0.1 and 0.01% m/m respectively. 2 mL from each calibration solution and unknown biodiesel sample were transferred into headspace vials, which were tightly crimped in order to prevent leaking.

Analytical Parameters

Table 1 includes selected instrument parameters for the TRACE GC Ultra and the TriPlus HS autosampler. These settings were used for all sample analyses.

TRACE GC Ultra

Injector	Split/Splitless (SSL)
SSL Mode	Split injection mode, split flow of 100 mL/min
SSL Temperature	160 °C
Carrier Gas	Helium, 1.5 mL/min, constant flow mode
FID	250 °C
Oven Program	50 °C (1 min) to 130 °C @ 10°C/min, final hold 0.5 min

TriPlus Headspace Autosampler

Incubation temperature	80 °C
Incubation time	40 min
Syringe temperature	85 °C
Injected amount	500 µL

Table 1: Selected instrument and method parameters for the TRACE GC Ultra and TriPlus HS

Results and Discussion

Figure 2 shows a typical chromatogram obtained from a biodiesel sample analyzed following the conditions reported above.

External standard calibration was performed by analyzing the three calibration solutions, and plotting the methanol contents vs. the respective peak areas. Figure 3 shows the calibration function calculated using linear regression. The correlation coefficient (R^2) for this calibration was 0.9999, demonstrating excellent linearity for this method.

Different unknown biodiesel samples from several sources were then analyzed, delivering results within the standard specification stated in EN 14214 (methanol < 0.2% m/m), as shown in Table 2.

After a long sequence of unknown biodiesel samples, "blank" headspace samples, prepared by filling the vials with nitrogen, were analyzed, demonstrating total absence of any carryover of MeOH, as well as of any interference compounds from vials or septa.

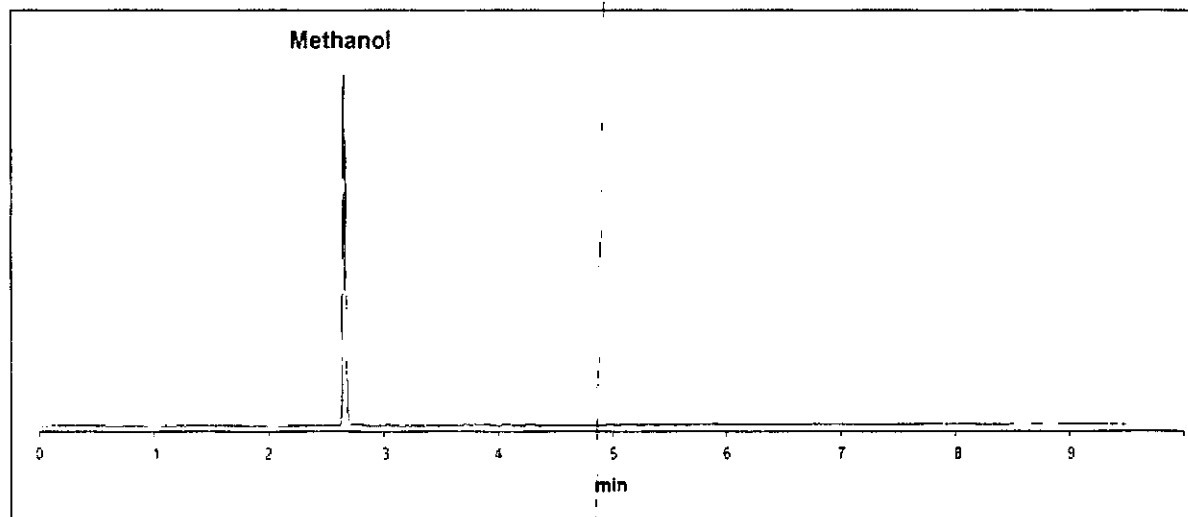


Figure 2: Biodiesel chromatogram showing methanol (headspace injection)

Finally, repeatability of the assay was tested by performing five consecutive injections for each biodiesel sample, resulting in excellent performance.

	Sample	% (m/m) MeOH	% RSD
1	Rapeseed biodiesel	0.18	1.4
2	Soybean biodiesel	0.05	1.9
3	Sunflower biodiesel	< 0.01	-
4	Mix 1-3 biodiesel	0.09	1.8
5	Mix 1-2 biodiesel	0.13	1.5

Table 2: Results for 5 biodiesel samples (5 runs each)

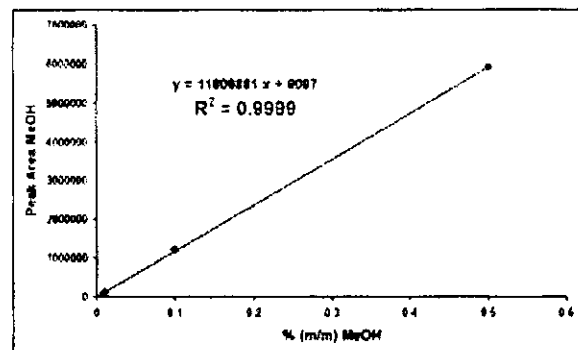


Figure 3: Methanol calibration curve, with levels at 0.01, 0.1, and 0.5% mass/mass (m/m)

Conclusion

The determination of residual methanol in pure biodiesel (B100) can be successfully achieved using the Thermo Scientific TRACE GC Ultra equipped with SSL injector and FID, and the TriPlus Headspace Autosampler, in full compliance with method EN 14110.

Although the procedure described here was based on external calibration and is preferred when a headspace autosampler is available or when a long sequence of samples needs to be analyzed, equivalent results can be obtained by applying the internal standard calibration procedure, either with the TriPlus autosampler or even manually.

As a further possibility, methanol content can be successfully determined by liquid injection of biodiesel diluted in *n*-Butanol, using a PTV Backflush system to vent out the heavier fraction without letting it enter the capillary column.

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Application Note SI-0773

Determination of Methanol Content in Biodiesel using Gas Chromatography with Headspace Sampling According to EN-14110

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Varian, Inc.

Introduction

The popularity and interest in biodiesel is significantly growing in many areas of the world and has become a commonly sought after alternative fuel source for use with diesel engines. Biodiesel is produced from vegetable oils or animal fats via transesterification using methanol to yield Fatty Acid Methyl Esters (FAME) and glycerine. The yield, pure FAME (once the glycerine and the residual methanol has been recovered/removed) is called B-100. In order for biodiesel to be used as a motor fuel or blended with petroleum diesel, it must conform to standard specifications (ASTM D 6751 or EN-14214). There are GC methods in use today to determine whether biodiesel conforms to the standard specifications. One of these methods, EN-14110, is used to determine the methanol content. EN-14110 is applicable for a concentration range from 0.01 % (m/m) to 0.5 % (m/m) methanol*.

Instrumentation

Varian 430-GC

- Injector: Split / splitless 1177, full EFC control
- Detector: FID, full EFC control

Headspace sampler

- QUMA, QHSS-40, sample loop mode

GC control and data handling software

- Varian Galaxie™ Chromatography Data System

Materials and Reagents

Column

- Varian Select™ Biodiesel for Methanol, 30 m x 0.32 mm x 3.0 μ m, CP-9083
- Fatty Acid Methyl Ester mixture (FAME) with a methanol content of <0.001 %

Sample Preparation

Calibration solutions

- Solution A: 0.5 % (m/m) methanol in FAME
- Solution B: 0.1 % (m/m) methanol in FAME
- Solution C: 0.01 % (m/m) methanol in FAME

A 1 mL aliquot was accurately weighed, and transferred into a 20 mL vial and then immediately capped.

Sample

A 1 mL sample was accurately weighed then transferred into a 20 mL vial and immediately capped.

Conditions

GC conditions

- Injector: 250 °C,
- Split rate: 50:1
- Detector: 275 °C, FID
- Oven: 80 °C (0.5 min. isothermal)
@ 20 °C/min to 160 °C (2 min)
- Carrier gas: 2.0 mL/min const. flow, Helium

QHSS-40

- Sample loop: 1 mL
- Vial/heating: 80 °C
- Equilibrium time: 45 min

Results and Discussion

All three calibration solutions were analyzed twice and a calibration curve was obtained. See Figure 1 for an overlay of the methanol peaks of the different calibration solutions. The calibration curve (Figure 2) shows excellent correlation with the method.

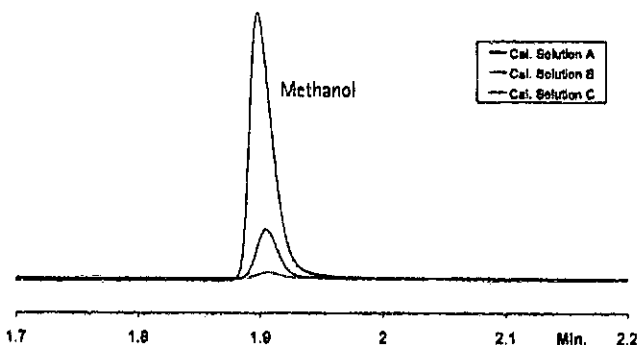


Figure 1. Overlay traces of calibration solutions.

*The method is not applicable to mixtures of FAME which contain other low boiling components.

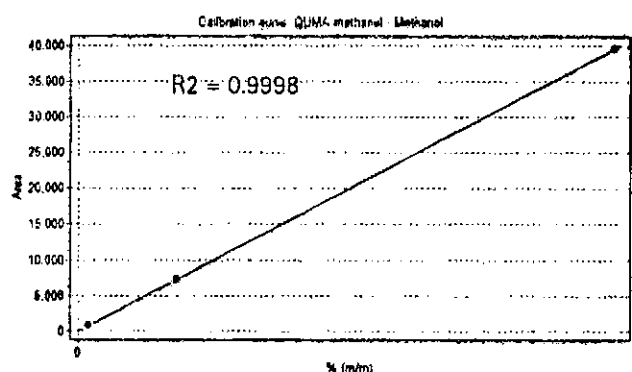


Figure 2. Calibration curve.

The correlation coefficient should be > 0.95. In this case the correlation coefficient was determined to be 0.9998.

A typical chromatogram of a biodiesel sample is shown in Figure 3.

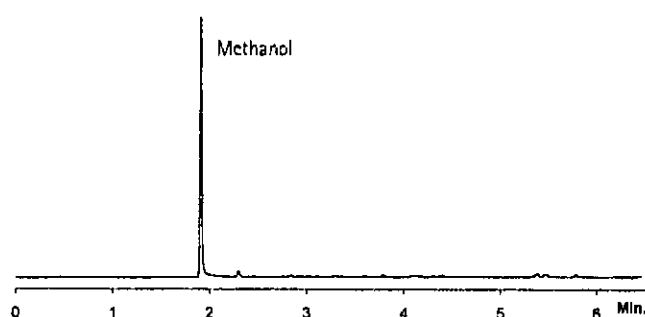


Figure 3. Typical headspace chromatogram of biodiesel.

Since biodiesel generally does not contain volatile components, other than methanol, identification and quantification is quite straightforward.

Methanol (mass %)	
N	15
Average	0.038
St. dev.	0.0007
RSD (%)	1.96

Figure 4. Repeatability figures.

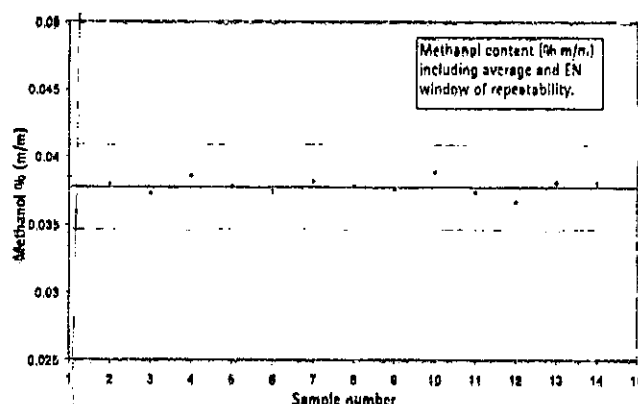


Figure 5. Repeatability values are within the specification boundaries established in EN-14214 as indicated by the red lines in the chart.

The methanol content of the biodiesel was 0.038 % (m/m) thus meeting the specifications set in EN-14214, (methanol content <0.2 %). Furthermore the repeatability figures indicated that the system was properly optimized for the analysis as seen in Figure 5, where the analyses trend line is well within the repeatability "window" set forth in the EN-14110 method. In Figure 5 this is visualized by adding the average line and the window of repeatability set in the EN-14110 method.

Conclusion

The GC Headspace system (Varian 430 Gas Chromatograph and a QUMA Headspace Sampler) was shown to be well suited for the determination of methanol content in biodiesel according to specifications outlined in EN-14110, and the biodiesel tested in this application note meets the specifications on methanol content set forth in EN-14214.

References

EN-14110 Fat and oil derivatives – Fatty Acid Methyl Esters (FAME) – Determination of methanol content.

EN-14214 Automotive fuels – Fatty Acids Methyl Esters (FAME) for diesel engines – requirements and test methods.

These data represent typical results.

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Determination of methanol in biodiesel by headspace solid phase microextraction

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Abstract

A new direct gas chromatography procedure (headspace solid phase microextraction) was developed for the quantitative determination of methanol in biodiesel. The analysis was performed by exposing a carboxen-polydimethylsiloxane SPME fiber assembly to the headspace of the biodiesel sample. The gas chromatography used a HP-5 capillary column and flame ionization detection. A polynomial relationship was observed between the methanol concentration and its peak area. This method showed good reproducibility (average relative standard deviation 7.06%) and recovery (average recovery 100.2%).

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Keywords: Headspace solid phase microextraction; Biodiesel; Methanol

1. Introduction

Biodiesel is a renewable fuel, generally consisting of a mixture of fatty acid alkyl esters, and is produced by reacting glycerides with an alcohol in the presence of a catalyst, as it can be seen in Fig. 1. Methanol is typically the alcohol used for the transesterification reaction. The catalyst can be alkali, acid or an enzyme. The stoichiometry of this reaction requires a 3:1 molar ratio of methanol to glycerides but in practice a higher amount of methanol is used (the molar ratio of methanol to glycerides is usually 6:1). The transesterification reaction is reversible and takes place stepwise. The initial triglycerides are transformed into diglycerides, then monoglycerides, and lastly glycerol. The excess of alcohol helps shift the equilibrium towards formation of fatty acid alkyl esters and glycerol. The reaction product is a mixture of fatty acid alkyl esters (biodie-

sel), tri-, di- and monoglycerides, glycerol, residual alcohol and catalyst. Most of these contaminants of the biodiesel need to be removed in order for the biodiesel to meet the American (ASTM D6751) and European (EN-14214) specifications. The excess of methanol is typically removed from the biodiesel by extraction and distillation (Ma and Hanna, 1999). The amount of methanol that can be present in biodiesel is regulated by the European biodiesel standards EN-14214 (test method EN-14110), the limit being 0.2% mass/mass (Knothe, 2006), and by the ASTM D6751-07a (test method D93 or EN-14110), the limit being 0.2% volume or at least 130 °C (<http://www.biodiesel.org>).

The quality of biodiesel depends on numerous factors, flash point being one of them. The flash point is directly affected by the quantity of alcohol in the biodiesel. The residual methanol in biodiesel can cause a lower flash point. High methanol content in biodiesel may cause a rapid deterioration of the rubber components of the vehicle's fuel system, so finding an accurate, reproducible, and direct method to determine the amount of alcohol (methanol) present in biodiesel is very important.

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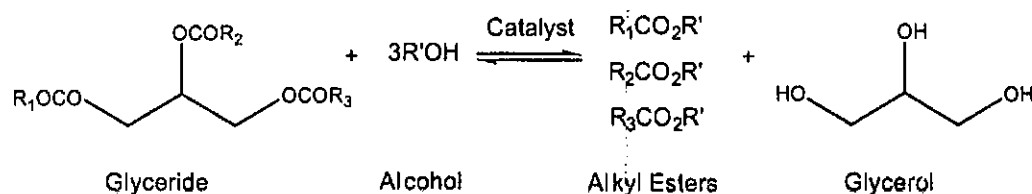


Fig. 1. General transesterification reaction.

One research group (Mittelbach et al., 1996) reported the determination of methanol in biodiesel by derivatizing it with *N,O*-bis-trimethylsilyl-trifluoroacetamide (BSTFA). They directly injected a mixture of rape-seed oil methyl esters and BSTFA in *N,N*-dimethylformamide into a gas chromatograph fitted with a DuraBond-5 capillary column and flame ionization and mass spectrometric detection. Other groups have used headspace solid phase microextraction (SPME) to determine the amount of methanol in other matrices, such as: pectin (Savary and Nunez, 2003), plant polysaccharides (Nunez et al., 2006), aspartame sweeteners (Sales and de Lourdes Cardeal, 2003), body fluids (Lee et al., 1999; Maleki et al., 2006), and air (Tuduri et al., 2002). While most of the headspace SPME analyses of methanol were performed with a carboxen-polydimethylsiloxane (CAR/PDMS) fiber assembly (Savary and Nunez, 2003; Lee et al., 1999; Tuduri et al., 2002), one research group (Tong et al., 2001) used pencil lead and reported good overall recoveries.

Solid phase microextraction was developed and patented by Pawliszyn and co-workers (1989). SPME uses a polymer coated fused silica fiber on which the analytes are adsorbed. The fiber is then directly inserted into a GC injector where the analytes are thermally desorbed and then the fiber can be reused. This technique has multiple advantages: no solvent is required, it is simple and fast, and there are two possible modes of analyte extraction, headspace and direct immersion. Also, SPME can be automated and can be coupled with GC, GC-MS, or HPLC.

One disadvantage is the limited lifetime of the fiber, which degrades with usage and can cause analyte peak tailing and co-elution. Headspace extraction is preferred over direct immersion because it prolongs the lifetime of the fiber due to the absence of contact between fiber and solution. A valuable feature of SPME is that different fiber coatings provide selective extraction of analytes from a mixture based on matching the properties of the fiber coating relative to the analyte polarity. The importance and use of this technique is increasing daily in many fields. SPME has been applied to analysis of environmental samples, flavor and food products, surfactants, forensic and toxicological analysis (Nunez et al., 2006; Sales and de Lourdes Cardeal, 2003; Lee et al., 1999; Maleki et al., 2006; Tuduri et al., 2002).

The unique feature of this research is the fact that it provides a direct method to rapidly and quantitatively determine the amount of free methanol in biodiesel samples.

The analytical method developed by our group is different from the current US standard for methanol determination, ASTM D6751 which uses the flash point (closed cup) measurement (test method D93) and from the European standard which uses the headspace GC method (test method EN-14110).

2. Methods

2.1. Chemicals and reagents

Optima-grade methanol was purchased from Fisher (Fair Lawn, NJ), 4A activated molecular sieves were acquired from Sigma-Aldrich (St. Louis, MO), anhydrous sodium sulfate (Na_2SO_4) was obtained from Merck (Darmstadt, Germany), and the biodiesel (B100) was supplied by the Petroleum Products Laboratory (Mississippi State, MS). The 10 mL SPME vials, fiber assembly, and the 75 μm carboxen-polydimethylsiloxane (CAR/PDMS) fiber, were purchased from Supelco (Bellefonte, PA).

2.2. Instrumental analysis

The analyte was adsorbed onto the SPME fiber and then thermally desorbed in the inlet of a Hewlett-Packard (HP) 6890N gas chromatograph (Palo Alto, CA) equipped with a split/splitless injection port and flame ionization detection (FID) system. The injector and detector temperatures were held constant during the analysis (200 and 300 $^\circ\text{C}$, respectively). The fused silica capillary column used for separation was a 30 m, 0.32 mm, i.d. 0.25 μm film thickness Hewlett-Packard HP-5 (5%-phenyl)-methylpolysiloxane. The GC oven was programmed as follows: the initial temperature of 40 $^\circ\text{C}$ was held for 4.0 min, increased to 120 $^\circ\text{C}$ at 10 $^\circ\text{C}/\text{min}$, and then increased to 200 $^\circ\text{C}$ at 20 $^\circ\text{C}/\text{min}$ and held at 200 $^\circ\text{C}$ for 1 min. Helium was used as carrier gas at a constant flow rate of 1.5 mL/min. The injector was operated in split mode (20:1 split ratio). The data were acquired using a HP-CORE ChemStation system.

2.3. Solid phase microextraction

An automated SPME system (CombiPAL, LEAP Technologies) was used with a 75 μm CAR/PDMS fiber assembly. The CAR/PDMS fiber is recommended for gases and low molecular weight compounds (MW 30–225). When new SPME fibers were installed they were conditioned in

the gas chromatograph (GC) injection port at 300 °C for 2 h, according to the manufacturer's recommendations.

The biodiesel (B100) used for calibration was washed three times with distilled water and dried with anhydrous sodium sulfate (Na₂SO₄) and molecular sieves. This procedure insured a practically complete elimination of methanol present in the initial biodiesel (B100). Standard solutions for calibration were prepared by spiking different amounts of methanol into the washed and dried biodiesel.

The 10 mL SPME vials each containing 1 mL of biodiesel solution were capped with Teflon lined septum caps and heated at 50 °C for 20 min, under constant stirring (500 rpm). The SPME fiber was then exposed to the headspace of the vial and the volatile compounds were adsorbed onto the fiber for 20 min at 50 °C, at constant temperature and stirring. Then the fiber was exposed for 2 min at 200 °C in the GC injection port for complete desorption and GC analysis of the analytes.

3. Results and discussion

The retention time of methanol (*R*_t = 2.15 min) was determined by direct injection of neat methanol. Washing the biodiesel (B100) three times with distilled water and drying it over anhydrous Na₂SO₄ and activated molecular sieves removed more than 99% of the initial methanol. The actual amount of methanol present in biodiesel after this procedure was 2.2E–04% mass/mass. This determination was performed using the above described headspace SPME and GC conditions. This washed and dried biodiesel was considered methanol-free and will be referred to as “reference biodiesel”.

Calibration curves were constructed by using standard solutions of methanol in reference biodiesel. The concentration of these standard solutions is expressed in percent mass (% mass/mass). Solutions were prepared by mass/volume and then converted to mass/mass units. Methanol/biodiesel (1 mg/mL) corresponds to 0.11% mass/mass (European standards). The average density of the biodiesel (B100) employed in this study (six replicates, relative standard deviation 0.12%) was determined to be 0.880 g/mL. The responses for calibration concentrations (shown in Table 1) were made with six replicates and the reproducibility was expressed as relative standard deviation (RSD). The range of RSD for these calibration points was from 3.41% to 10.36% and the average RSD was 7.06%.

In the concentration range from 0.0057 to 0.057 mass%, the results fit a second order polynomial curve ($y = 2E-07x^2 + 1E-04x - 0.0011$) (Fig. 2). For higher methanol concentrations, a third order polynomial curve must be used (Fig. 3).

Three concentrations of methanol in reference biodiesel were used to check the reproducibility of this headspace SPME method. The concentrations were 0.057, 0.11, and 0.23 mass%. For each concentration, a set of six replicates was recorded and the relative standard deviation was 1.00% for the 0.057 mass% solution, 1.52% for the

Table 1
Average values of RSD (*n* = 6) for calibration concentrations

Methanol/biodiesel concentration (% mass/mass)	Average RSD (%)
0.0057	9.94
0.011	9.64
0.023	9.92
0.034	10.36
0.045	10.33
0.057	5.52
0.11	4.42
0.23	3.41
0.57	3.43
1.14	3.67
1.70	6.67
2.27	7.47

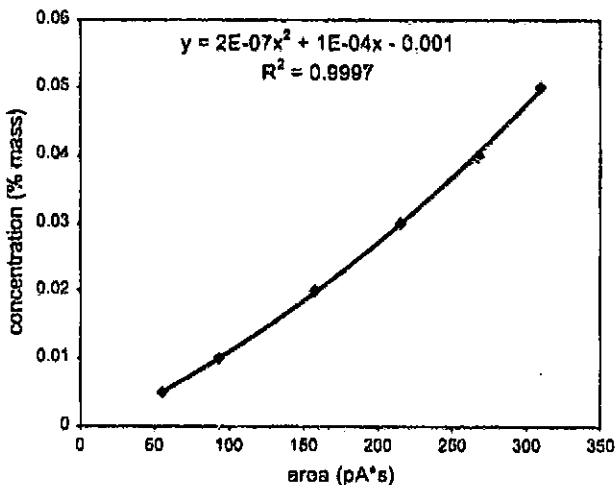


Fig. 2. Methanol in reference biodiesel – 0.0057% to 0.057% mass/mass.

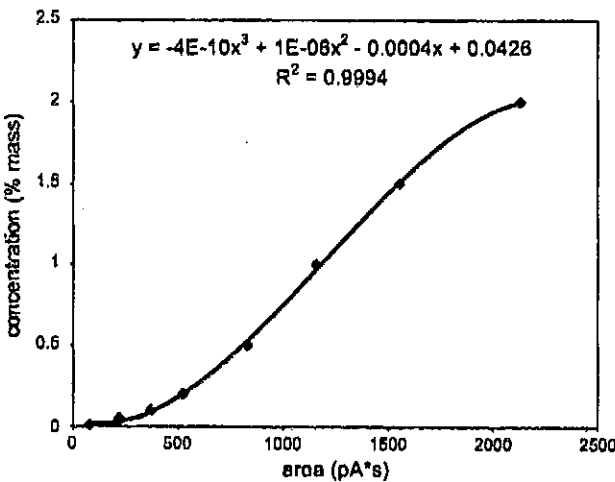


Fig. 3. Methanol in reference biodiesel calibration curve – average of six series.

0.11 mass% solution, and 2.01% for the 0.23 mass% solution. The recovery was also excellent, ranging from 97.43% to 101.55% with an average of 100.20% and a relative standard deviation of 1.60% (*n* = 6).

This headspace SPME method was used to analyse 13 regulatory biodiesel (B100) samples obtained from the Petroleum Products Laboratory (Mississippi State, MS). The results are summarized in Table 2. All the samples in this table, except number 10, 11 and 13 passed the European EN-14110 standard.

The European EN-14110 test method uses biodiesel (B100), which is heated at 80 °C in a hermetically sealed vial for 45 min and then a defined amount of gas phase is injected into the GC/FID by means of a preheated syringe. This headspace method can be manual or automated and it uses 2-propanol as internal standard. An external calibration can also be used if the headspace procedure is automated (BS EN-14110, 2003).

The analytical method developed by our group uses an automated headspace SPME assembly, which provides a better reproducibility than its manual version and allows multiple and continuous sample processing. The capillary column used for methanol determination is HP-5 fused silica (5%-phenyl)-methylsiloxane, which provides reliable analysis and is suitable for a wide range of applications. EN-14110 uses 2-propanol as internal standard and is applicable for the 0.01–0.5 mass% concentration range. Our group considered a broader range of concentrations, varying from 0.0057 to 2.27 mass%, and no internal or external standard was required.

The ASTM D6751 uses the flash point method (test method D93) to control the amount of alcohol in biodiesel. This method uses a Pensky–Martens closed cup tester in which about 70.0 mL of sample is heated at constant temperature rate and stirring. The test flame is applied into the cup and the temperature at which the vapors above the sample ignite is recorded. The limit for the flash point temperature is at least 130 °C (<http://www.astm.org>).

The new analytical method developed is a simple, direct and reliable alternative to the two standard methods for methanol determination in biodiesel. It needs less sample volume than D93 test method, which is an indirect method,

it uses a general purpose GC capillary column, so the same HS-SPME assembly can be used for different types of analysis, and is the first HS-SPME method used to extract methanol from biodiesel as matrix.

4. Conclusion

A new, direct and quantitative method for determining the amount of methanol in biodiesel was developed. Headspace SPME proved to be reproducible and sensitive, allowing analysis for concentrations of methanol in biodiesel well below those imposed by the standard specifications. There is no need for using an internal standard or derivatization. The sample volume required is 1.00 mL as compared to the flashpoint method that requires at least 70.00 mL. This method was used for analyzing biodiesel samples from various producers. To our knowledge it is the first literature report that uses headspace solid phase microextraction to determine the amount of methanol in biodiesel as a substrate.

Acknowledgements

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Table 2

Methanol concentration in biodiesel samples from Petroleum Products Lab

Sample no.	Sample name	Methanol concentration (% mass/mass)
1	2006-93A	0.0085
2	2006-93H	0.12
3	2006-95A	0.015
4	2006-107A	0.081
5	2006-108A	0.043
6	2006-108B	0.0022
7	2006-108C	0.073
8	2006-108D	0.16
9	2006-108E	0.095
10	2006-109B	0.61
11	2006-111A	0.72
12	2006-111B	0.15
13	2006-111C	0.25

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Head-space analysis in modern gas chromatography

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Recent developments and trends in the use of headspace sampling with gas chromatography (GC) are reviewed and discussed. A brief overview of headspace analysis techniques and theory references is provided, followed by publication trend data on the various headspace-analysis techniques. The techniques described include static and dynamic headspace extraction, solid phase micro-extraction (SPME) and several additional techniques now receiving attention. Examples of applications in environmental, clinical, forensic, biological, food, flavor and pharmaceutical analysis are provided. Research in headspace-GC is very active and growing, with new applications being reported continually. © 2002 Published by Elsevier Science B.V. All rights reserved.

Keywords: Gas chromatography (GC); Headspace; Sample preparation; Solid phase micro-extraction (SPME)

1. Introduction

When GC was introduced 50 years ago [1], sampling and sample introduction were immediately recognized as crucial issues in successful analysis. Because for classical GC generally samples must be dissolved in organic solvents injections and, for modern capillary GC, be dilute, some sample preparation is required in

nearly all GC analytical methods. Because GC requires that samples be volatile, it is a natural tool for the analysis of vapor-phase mixtures. Headspace analysis is generally defined as a vapor-phase extraction, involving the partitioning of analytes between a non-volatile liquid or solid phase and the vapor phase above the liquid or solid. It is expected that the vapor-phase mixture contains fewer components than the usually complex liquid or solid sample and that this mixture is transferred to a GC or other instrument for analysis. There are a number of techniques for sampling headspace vapors and introducing them to a GC. In this article, recent developments in the use of headspace sampling with GC are reviewed, with application examples drawn from the recent literature. While this review cannot be fully comprehensive, we have attempted to sample some of the most interesting current work employing headspace sampling with GC, including recent trends and expected future developments.

2. History and scope

The idea of analyzing samples of the vapor above a solid or liquid for their organic content originates long before the development of GC. Perhaps the first specific mention of the analysis of the vapors above a liquid in the chemical literature occurred with the paper of Harger, Bridwell and Raney in 1939, in which they proposed determination of the alcohol content of

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aqueous solutions [2]. The first reported use of static headspace with GC occurred in 1958 [3] and the first widely-read use of dynamic headspace ("purge and trap") with GC occurred in the 1970s soon after the introduction of Tenax as a commercial adsorbent [4]. In the intervening decades, techniques for both static and dynamic headspace sampling in combination with GC have evolved significantly and the theory of headspace sampling and transfer of the samples to the GC has been well-developed. Several texts provide excellent overviews of headspace-extraction theory and explanations of the fundamentals of headspace-GC analysis and instrument configurations, so these are not explored in-depth here [5–8]. In 1999, Kolb provided an excellent review of the principles and instrumentation of headspace-GC [9]. More recently, Ettre has provided an especially straightforward review of the principles of static headspace-GC [10].

A decade ago, there were essentially two techniques that could be described as headspace-GC: static (vapor-phase extraction) and dynamic (purge and trap). Today, there are several more, as techniques have become miniaturized and automated. The classical technique and the simplest way to try headspace analysis for the first time is to seal the sample in a gas-tight vial, using a closure with a septum and to use a gas-tight syringe to sample the vapors

within the vial. This is called static headspace sampling and numerous instruments are available for its automated application. While most modern headspace-GC instruments employ static sampling, they typically replace the syringe with a heated transfer line and they pressurize the sample vial above the capillary column head pressure, which allows for more inert sampling, rapid sample transfer and ready equilibration, for interfacing the sampling device to the GC. The configuration of a modern static headspace-GC sampling system is shown in Fig. 1.

If the vapor phase flows through the sample and is later trapped on a sorbent, or somehow collected, the technique is termed dynamic headspace extraction. The most important example is the classical purge-and-trap technique. Dynamic headspace is often used when a large degree of analyte concentration is needed. In his review, Kolb provided a number of diagrams of common instrumental configurations for several dynamic headspace-GC systems [9]. A dynamic headspace-GC system that uses a cryogenic trap to re-focus the analyte bands for separation is shown in Fig. 2. There are numerous ancillary techniques, such as multiple headspace extraction, that are based on these classical ideas, and are described in the aforementioned references. Recently, sorbent and membrane techniques have been used for the analysis of headspace samples. Sorbent-based

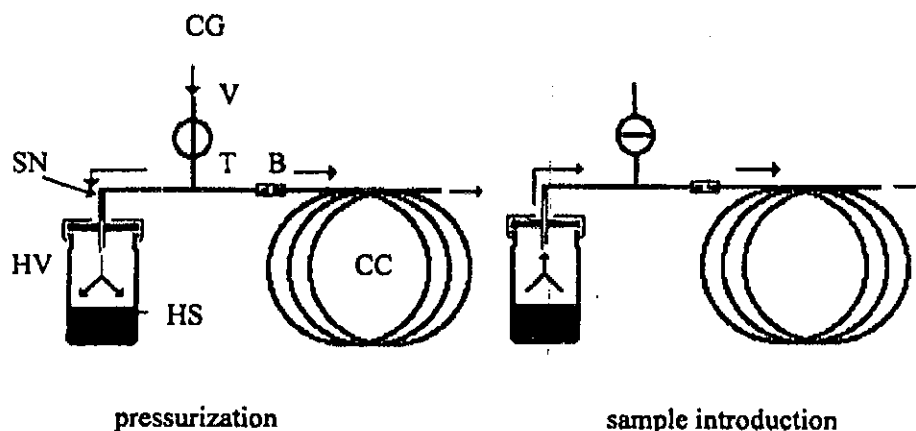


Fig. 1. Schematic diagram of a pressure balances static headspace system. Sample vial is first pressurized and equilibrated with carrier gas. Pressurized sample is then transferred to the GC column. CG=carrier gas, V=solenoid valve, SN=sampling needle, HV=headspace vial, HS=headspace sample, T=fused silica transfer line, B=butt connector, CC=capillary column. Reprinted with permission from [9].

methods have seen a renaissance since the introduction of SPME by Pawliszyn and co-workers in 1990 [11] and the introduction of headspace-SPME in 1993 [12]. There are a number of texts now available describing SPME theory and applications [13-15]. Even more recently, membrane technology has been used in the dynamic extraction of headspace vapors [16]. Headspace solvent microextraction, in which a small drop (a few microliters) of solvent is used for headspace extraction, was introduced in the late 1990s [17].

Headspace sampling is tremendously widely applied in numerous scientific fields. In preparing this article, a literature search on Sci Finder Scholar (Chemical Abstracts Service, Cleveland, OH, USA) using the term "headspace" yielded approximately 8700 articles over the past 75 years or so. If this broad search is narrowed to include only references involving GC, the number narrows to about 6000 references, indicating that 65% of all papers employing the concept "headspace" also include GC. Of these 6000 articles, about 1400 involve dynamic headspace-GC techniques. Surprisingly, over 510 papers involve headspace-SPME, all published since

1993. Headspace analysis using membrane sampling was first published in 1977, with 62 publications in total.

A plot of publication trends is shown in Fig. 3. Dynamic headspace and SPME represent the largest categories besides static headspace. The publication numbers for other methods, such as membrane extraction, while many are rising, are still too small to provide meaningful comparison.

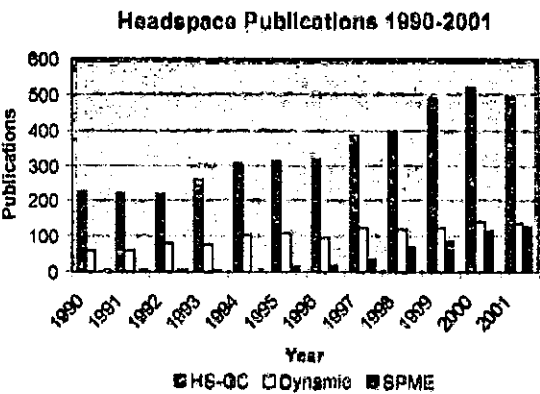


Fig. 3. Trends in headspace-GC publications: 1990-2001. HS-GC=all papers including the concepts "headspace" and "GC", Dynamic=papers containing the concepts "headspace" and "dynamic", SPME=papers containing the concepts "headspace" and "SPME".

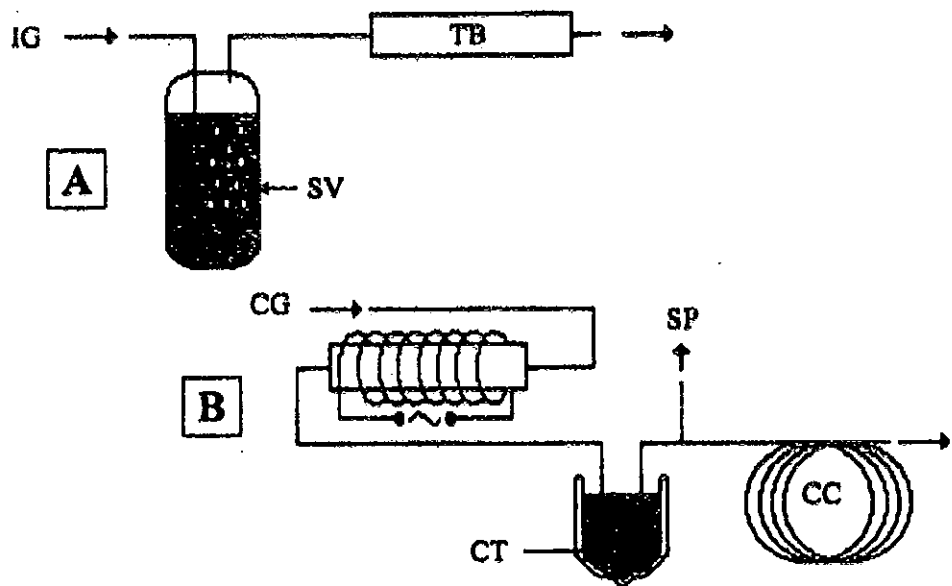


Fig. 2. Schematic diagram of a dynamic headspace-GC system with cryogenic sample re-focusing. A: Sample is purged with gas, and the volatiles are collected in a sorbent trap. B: The sorbent trap is heated and purged with carrier gas to desorb the analytes; bands are re-focused in the cryogenic trap. IG=inert purge gas, CG=carrier gas, TB=sorbent trap, SV=sample vessel, CC=capillary column, CT=cryogenic trap, SP=inlet splitter. Reprinted with permission from [9].

It is interesting to note that much of the recent upswing in overall headspace-GC publications can be attributed to the rise of headspace combined with sorbent-based trapping techniques, such as purge and trap and SPME.

3. Static headspace sampling

Static headspace sampling is employed with GC in numerous fields and with a variety of applications. It has been a primary tool for analysis of volatile organic compounds in environmental and flavor and fragrance analysis for decades. Static headspace sampling, because of its limited sensitivity, is mostly employed for applications in the high-ppb to percent concentration ranges. In the past decade, there has been a considerable rise in the use of static headspace analysis in other fields, including pharmaceuticals, clinical and biological analyses. Despite the rise of other techniques, as described later in this review, static headspace remains the most readily automated and validated of all headspace sampling techniques. As examples of its versatility, a selection of static headspace-GC applications in several industries is given below.

The reports by Camarasu [18] and de Smet et al. [19] provide validated examples of the application of static headspace-GC to the analysis of residual solvents in pharmaceuticals, which is perhaps the most common application of GC in that industry. In a new development, Hong and Altorfer developed a promising miniaturized static headspace technique for solvent analysis in drugs, which reduced sample sizes by about a factor of 10 over traditional methods and provided some validation data [20]. In addition to solvent analysis in pharmaceuticals, there has recently been a tremendous upsurge in the use of static headspace in clinical, forensic and biological analysis. The proliferation of headspace articles in non-traditional separations and analytical journals shows clearly that static headspace sampling has matured into widespread use by non-specialists.

Headspace-GC is rapidly becoming a tool of choice for the analysis of small molecules that

are reaction products in a huge variety of metabolic processes, or toxins or other substances of interest in biological systems. A wide variety of examples have appeared recently, a few of which are presented here. In forensic analysis, headspace-GC is used for the analysis of alcohol and other low molecular-weight materials in blood, as well as in arson and fire investigations [21]. Moriya and Hashimoto have provided a thorough description of potential errors in the assessment of cyanide levels in blood using headspace-GC [22] and Jones and Holmgren thoroughly discussed uncertainties in the determination of blood-ethanol concentrations by analysis of vitreous humor [23]. Each of these articles discussed some aspect of error analysis in static headspace techniques, which is critical in forensic analysis. It is seen that its excellent sensitivity, reproducibility and stability make static headspace sampling a method of choice in forensic analysis.

In clinical analyses, it is especially interesting that headspace methods are appearing in non-analytical science journals, indicating its deep penetration beyond use by traditional specialists. For example, Sharp and Dautbegovic studied the stability of ether in blood as part of the investigation of an ether-related suicide [24]; Sarkola and Eriksson examined the production and metabolism of endogenous ethanol and methanol, using static headspace-GC as the main analytical tool [25], and Haze et al. used headspace GC/MS to analyze body odor in human subjects aged 25-75 for 2-nonenal content and suggested a correlation between age and the amount of 2-nonenal in body odor [26].

Static headspace-GC has been used for the analysis of natural aromas and odors in several industries. This is another area in which many papers are being published in far-reaching areas. For example, in food analysis, Panosyan et al. qualitatively and quantitatively determined the volatile components in brandy by static headspace GC-MS [27], and Schiberle and Steinhaus used headspace-GC along with dilutions of the sample and isotope dilution-MS to determine the odor-active components in fresh and processed hops [28]. In natural products analysis,

Royer et al. developed an automated headspace-GC method for the determination of dithiocarbamates in plant matrices and found that the method was much simpler than classical techniques [29].

These examples further emphasize the wide application of static headspace-GC and its use by specialists and non-specialists alike. The main limitation of classical static headspace-GC is that sensitivity is limited, compared to dynamic techniques, such as purge and trap and to techniques involving on-line concentration such as SPME.

4. Dynamic headspace purge-and-trap sampling

Dynamic headspace sampling involves the passing of carrier gas through a liquid sample, followed by trapping of the volatile analytes on a sorbent and desorption onto a GC. This is a well-known, validated technique, which is also routinely performed by non-specialists, and it is the method of choice for analysis of extremely low (ppb and ppt) concentrations of volatile

organic compounds in aqueous matrices. For dynamic headspace-GC techniques, publication trends continue to rise, but perhaps not at the rapid rate of headspace-SPME and some of the other sorbent-based techniques.

As with static headspace sampling, recent applications are seen in a very wide variety of industries, including environmental contaminants, foods and aromas. For example, Olliver and Guerre used dynamic headspace-GC to measure benzene hydrocarbons in virgin olive oil at levels below 0.1 mg/kg [30]. Martin et al. have developed a dynamic headspace-GC method for the determination of aroma compounds in cheese curd, cultured with various yeasts and bacteria. They found that dynamic headspace-GC provided the best quantitation of the aroma compounds, which are often at very low concentrations [31]. The second example further demonstrates the maturity of purge-and-trap sampling for GC, as the work has progressed from basic developments to its use in solving increasingly difficult problems.

Roose and Brinkman provided an interesting example, showing both the sensitivity and versatility of dynamic headspace sampling, coupled

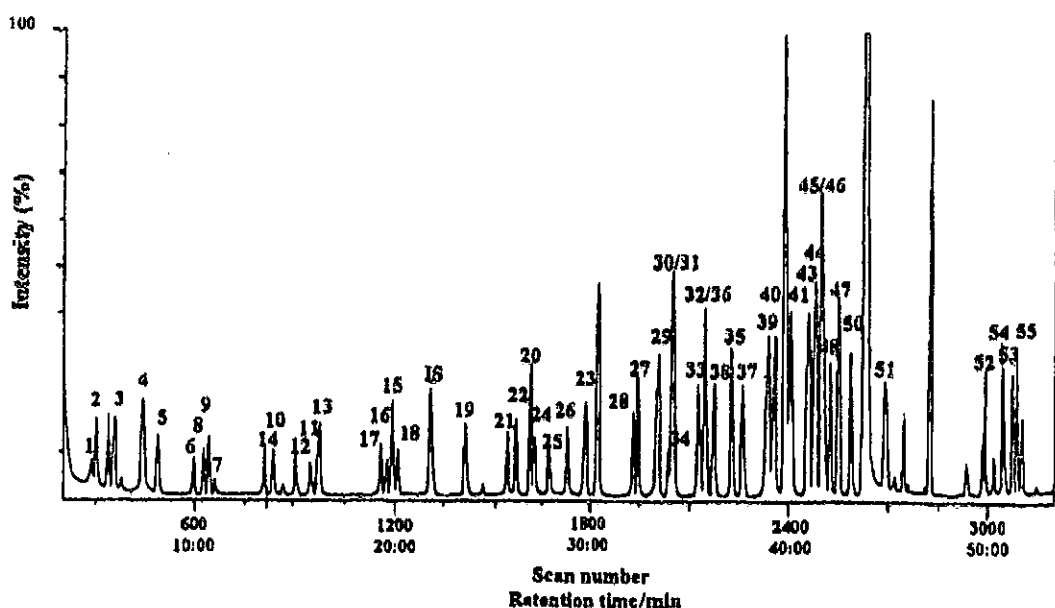


Fig. 4. Full-scan GC/MS separation on a 60m×0.25mm ID×1.4 μm film thickness DB-VRX column, of 56 volatile organic contaminants obtained by purge-and-trap headspace sampling from an aqueous sample. Reprinted by permission of the Royal Society of Chemistry from [32].

with GC/MS [32]. For this paper, they analyzed extracts of marine organisms for volatile organic contaminants at ppt levels, using an on-line purge-and-trap sampler. They were able to determine 55 compounds in a total analysis time of about an hour. An example chromatogram of volatile organic contaminants in water obtained using their method is shown in Fig. 4.

5. Headspace-SPME

Headspace-SPME, in which a coated fused-silica fiber is used to trap and concentrate analytes from a static or dynamic headspace process, was developed in 1993 and has experienced the strongest growth in research interest over the past decade. This technique can also now be automated routinely and has been validated for numerous applications. SPME is generally applied to samples with concentrations in the ppb-low ppm range, while some applications in the ppt range have been described [13]. SPME has shown pleasantly surprising versatility and has been employed for a tremendous variety of analytical problems, as evidenced by the recent applications shown here. Fig. 5 shows a schematic diagram of a headspace-SPME procedure, including extraction, followed by desorption into classical splitless GC inlet.

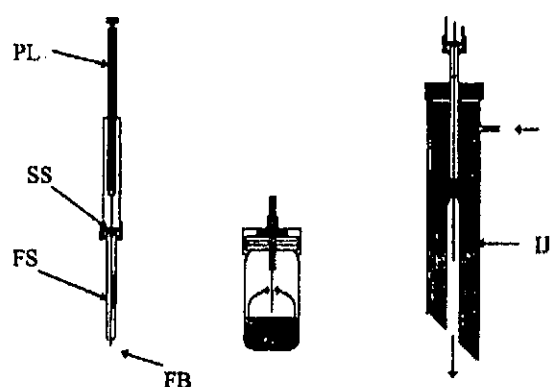


Fig. 5. Schematic diagram of the steps in a headspace-SPME analysis (from left to right): SPME fiber holder (FB=coated fused silica fiber, FS=syringe needle, SS=sealing septum, PL=plunger); headspace absorption of analytes into SPME fiber coating; and, desorption into heated GC inlet (U). Reprinted with permission from [9].

Researchers in several areas, including environmental, pharmaceutical, food and natural products analysis, have enthusiastically used headspace-SPME for many difficult analyses. The examples presented here provide a sample of the hundreds of publications that have recently appeared in a relatively short time.

In an environmental application, Cancho, Ventura and Galceran used *in-situ* derivatization followed by headspace-SPME-GC-ECD to determine aldehydes in treated drinking water. After optimizing a number of experimental parameters, they found this method to be comparable to liquid-liquid micro-extraction, proposed in US EPA method 556 [33]. This paper follows the example of much of the literature in HS-SPME, as it makes a thorough comparison to a compendial method employing a traditional extraction technique.

Recently, in residual solvent analysis of pharmaceuticals, Coran et al. described a headspace-SPME-GC method, using isotope dilution (without mass spectrometry) for several solvents in ketoprofen raw material and tablets [34], and Scypinski and Smith reported on the comparison of SPME with classical methods for the analysis of solvent in several pharmaceuticals [35]. Fig. 6 shows an example of residual solvents separation, using the isotope dilution method of Coran et al., in which they used a 100µm film thickness polydimethylsiloxane fiber and isothermal GC. This chromatogram shows that headspace-SPME-GC can be a powerful tool in resolving and quantitating very closely related compounds.

In natural products analysis, Shang, Hu and Deng used headspace-SPME-GC to determine the aroma components of fresh, frozen and withered *Michelia alba* flowers and were able to determine the differences in the compounds present in each case [36]. Yang, Huang and Smentena optimized a headspace-SPME method for determination of the volatile components of tobacco [37].

In food and flavor analysis, headspace-SPME was used by Pinho et al. to determine the components in the volatile fraction of ewe cheese [38], and by Alonso and Fraga to quantitate

flavor compounds in yogurt [39]. Characteristic of SPME methods, these papers describe methods that generated large amounts of information from relatively straightforward procedures.

Some users have complained of difficulties in method validation with SPME, including problems with extraction reproducibility as a result of instrumental issues, such as difficult automation, sample agitation, temperature control and fiber aging and reproducibility. But, recent advances in instrumentation should propel headspace-SPME into the mainstream of routine GC analysis.

Several options for automating and rapidly optimizing SPME extractions are now available. A recent research example is the development by Pham-Tuan, Vercammen and Sandra of new robotic arm for use with SPME that allows for easy transportation of the sampler, simplifying analysis from many sizes and types of sample containers and at remote locations [40]. Com-

mercially, there are several SPME auto-injectors available.

An SPME variant, stir-bar sorptive extraction (SBSE) was demonstrated recently [41]. In one application, Bicci et al. used headspace-SBSE to analyze the flavor components of various coffees [42]. This new technique employs a phase-coated stir bar that is placed in the headspace of the sample, then transferred by thermal desorption to a GC inlet. SPME and SBSE provide examples of an interesting trend in the increased use of both selective and non-selective sorbent traps as concentrators in both static and dynamic headspace sampling.

6. Further new techniques and developments

While static, dynamic and SPME headspace-GC techniques have reached levels of maturity

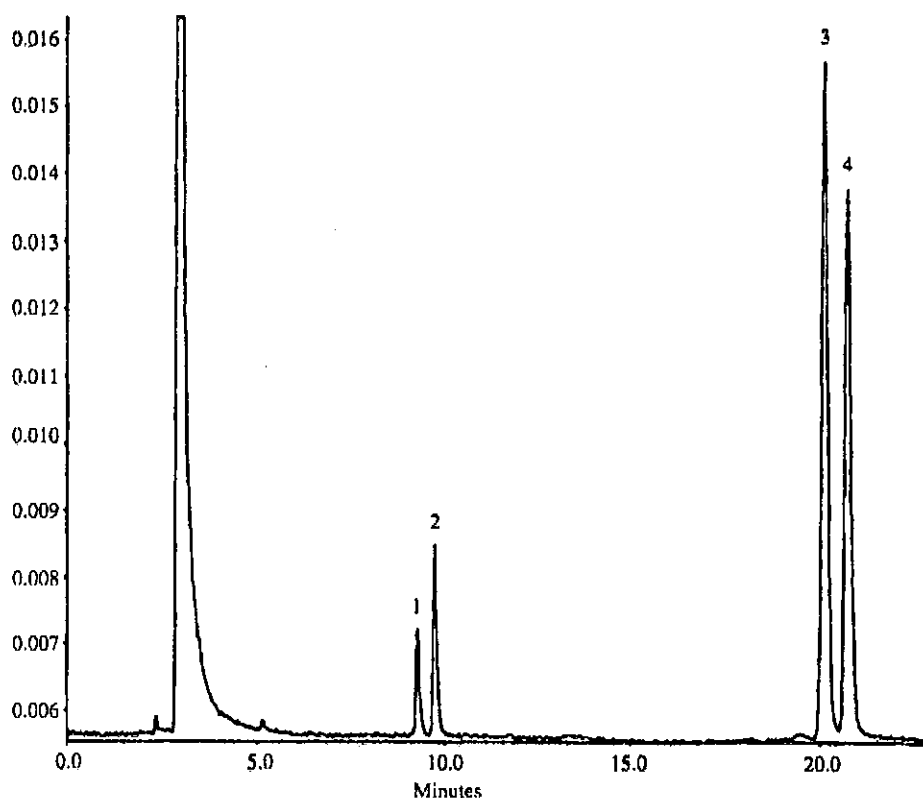


Fig. 6. Headspace-SPME-GC-FID chromatogram showing separation of hydrocarbons and their deuterated analogs. 1= d_{12} -cyclohexane, 2=cyclohexane, 3= d_8 -toluene, 4=toluene. Reprinted with permission from [34].

that make them suitable for use routine analysis by specialists and non-specialists alike, there are several newer techniques that are receiving attention in the literature and that have tremendous potential. These include membrane extraction techniques and headspace solvent micro-extraction [43]. Further, there have been numerous recent developments in the classical techniques that show that, while they are mature, research and development of new techniques and instrumentation is and should remain active in the near future.

Headspace-membrane extraction techniques have been used by several authors for environmental and process-stream monitoring, which seems to be their major application. Generally, these techniques have been applied with custom-built instrumentation for specific analyses. Membrane extraction has been employed for headspace since 1977, but, with a total of only 62 publications, research progress has been relatively slow in comparison with that of SPME. Given the successes shown in the examples presented here, increased attention to these techniques, which are readily automated and interfaced for field analyses, is warranted.

Minicola et al. used membrane extraction to analyze semi-volatile components, such as *n*-decanol, naphthenes and paraffins from aqueous process streams [44], and Ciucanu and Pawliszyn have developed a field-portable device for continuous monitoring of air, based on membrane extraction with a sorbent interface (MESI) [45].

There continues to be strong activity in the development of new instruments and measurement techniques around classical headspace-GC. Recently, Hoke, Laconto and McGrath used an indirect static headspace-GC method to analyze small amounts of water adsorbed on metal surfaces [46], and Markelov and Ber-shevits developed vapor-phase sweeping, which allowed the complete removal of the vapor phase from an equilibrated headspace vial, and showed excellent results for several ultra-trace analysis applications [47]. Reaction headspace was used by Chai, Luo and Zhu to determine carbonate in several matrices [48]. Ojala et al.

used a new dynamic headspace sampling technique, purge and membrane extraction in combination with GC and MS for the analysis of volatile organic contaminants from soil [49]. Overton, et al. described a field-portable GC, which was optimized for rapid analysis and included a sorbent-based sampling system that is suitable for headspace samples [50]. Giardina and Olesik [51] examined the use of low-temperature, glassy, carbon films with SPME and found that they were especially selective for molecules with high polarizability and large cross-sectional surface area. These examples show that the development of incremental improvements to the family of headspace-GC techniques is and is expected to remain strong in the future. Further, the commercial instrument vendors are continually updating their product lines, as the market for headspace-GC instrumentation remains very active.

7. Trends and conclusions

There are several interesting observations that can be made by examining the trends presented here. First, headspace-GC remains of wide research interest in many fields. In environmental analysis, there have been a number of papers addressing issues, such as field portability and multi-dimensional techniques, in which headspace sampling is used, but is not the primary focus of the work, while the number of papers employing the classical headspace techniques appears to be decreasing. However, the volume of environmental research using the newer techniques is increasing rapidly, indicating a continued strong interest in this area.

The relatively small number of papers directly related to pharmaceutical analysis is also interesting, given the strongly regulated environment.

In both these areas of application, this could be because widely published, applied and validated compendial analytical methods already exist.

The large number of clinical, biological and natural products applications is pleasantly surprising.

A number of journals outside of the traditional realm of separation scientists have been referenced here for a variety of applications. They show that headspace sampling has matured and is now being used as an effective research tool by non-specialists in separations and that separation scientists should look as widely as possible in their own literature searching.

The large number of food and flavor applications is not as surprising, as headspace sampling has been a staple analytical technique in these industries for many years. It is interesting to note that artificial noses and olfactometry are discussed in several of the papers, indicating a strong interest in miniaturized instrumentation.

There is a strong interest in the use of headspace-GC for both custom and routine applications.

Theoretical research and new developments in instrumentation have been active since the inception of headspace as an analytical technique and this has not changed.

Trends in instrumentation indicate focus on improved automation and ruggedness, field portability and novel selectivity for specific applications.

Many researchers are still using headspace-GC for fundamental chemical and physical property measurements.

In 2002, headspace sampling remains one of the premier sampling technologies for use with GC. It is perhaps the most versatile of all sampling techniques available for capillary GC, with active research being conducted by numerous researchers across many areas of science.

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Simple and solvent-free methodology for simultaneous quantification of methanol and acetic acid content of plant polysaccharides based on headspace solid phase microextraction-gas chromatography (HS-SPME-GC-FID)

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Abstract

A simple and solvent-free methodology is proposed for the simultaneous determination of methanol and acetic acid content of any type of plant cell wall polysaccharides. The methanol and acetic acid, released by saponification, are extracted from the headspace of the aqueous solution by solid phase microextraction (HS-SPME) using a DVB/Carboxen/PDMS fibre. The analytes are separated by gas chromatography and detected using a flame ionization detector (GC-FID). The quantification of methanol and acetic acid is done using external calibration curves. A linear relationship between the concentration of methanol (40–100 mg L⁻¹) and acetic acid (25–105 mg L⁻¹) and their GC peak area was observed ($r^2 = 0.987$ and 0.988 , respectively) with a reproducibility of 10%. HS-SPME-GC-FID revealed to be a clean, simple, fast and reliable methodology for the determination of the methanol and acetic acid content of cell wall polysaccharide extracts.

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Keywords: Methanol; Acetic acid; Solid phase microextraction; Headspace analysis; Plant polysaccharides

1. Introduction

Acetylation of plant cell wall polysaccharides decreases the interpolymer binding, leading to weak and unstable cell walls (Aspinall, 1980; Kunzek, Kabbert, & Gloyna, 1999; Pilnik & Rombouts, 1985; Van Buren, 1979; Wakabayashi, 2000). In pectic polysaccharides, the number and distribution of methyl and acetyl groups of galacturonic acid residues influence widely their ion-exchange properties, water-binding capacity, cross-linking through calcium ions and hydrogen bonding. Consequently, these substitutions are key parameters for understanding and predicting the role of these polysaccharides within the cell walls, namely, their contribution to the texture of fruits during ripening, storage and processing (Voragen, Pilnik, Thibault, Axclos, & Renard, 1995).

Several methods have been developed to quantify the methyl groups in pectic polysaccharides. The Food Chemical

Codex (1981) proposes a titrimetric method for determination of the degree of methylesterification (DM) of pectins. This procedure involves titration with sodium hydroxide of a polysaccharide suspension before and after alkali hydrolysis. Because acetyl groups are usually present, the DM value can be only obtained after correction for the acetyl groups by a separated specific determination. Another possible disadvantage of this method is the need of relatively large quantities of sample (ca. 30 mg). The amount of methanol can also be quantified by colorimetry (Wood & Siddiqui, 1971), alcohol oxidase enzymatic assay (Klavons & Bennett, 1986) and capillary zone electrophoresis (Jiang, Liu, Wu, Chang, & Chang, 2005). Direct determination of DM using ¹H NMR (Grasdalen, Bakøy, & Larsen, 1988; Renard & Jarvis, 1999) and FT-IR (Barros et al., 2002; Chatjigakis et al., 1998; Gananasambandam & Proctor, 2000; Monsoor, Kalapathy, & Proctor, 2001) is also a possibility. However, for the simultaneous determination of methanol and acetic acid, gas chromatography (Huisman, Oosterveld, & Schols, 2004; McFeeters & Armstrong, 1984; Waldron & Selvendran, 1990) and HPLC (Levigne, Thomas, Ralet, Quemener, & Thibault, 2002; Voragen, Schols, & Pilnik, 1986) are the most frequently used methodologies.

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Solid-phase microextraction (SPME) is a sample preparation technique that is simple to use, relatively fast and does not require solvent extraction (Arthur & Pawliszyn, 1990; Zhang & Pawliszyn, 1993). It has been successfully applied to a wide variety of compounds, in particular for the extraction of volatile and semi-volatile compounds from environmental, biological and food samples, since SPME is available with various sorbent materials and various coating thickness (Katnoka, Lord, & Pawliszyn, 2000; Mester, Sturgeon, & Pawliszyn, 2001). The use of solid phase microextraction (SPME) coupled with gas chromatography–mass spectrometry (GC–MS) was also proposed for the simultaneous determination of the degrees of methylation and acetylation in 1 mg of fruit and vegetable pectic polysaccharides, using a Carboxen/PDMS SPME fibre (Savary & Nuñez, 2003). In this study, deuterated isotopomers were used as internal standards and for construction of calibration curves were used concentrations between 0.1 and 8.0 mmol mL⁻¹ (3–260 mg L⁻¹) for methanol and 0.05 and 2.0 mmol mL⁻¹ (3–120 mg L⁻¹) for acetic acid.

In order to propose a simple methodology and to avoid the use of a mass detector and deuterated species, in this work it is proposed a new methodology for the simultaneous quantification of methanol and acetic acid released from polysaccharides after saponification. The headspace of the samples were analysed by SPME using a GC equipped with a flame ionisation detector (FID). Different coating fibres (a DVB/Carboxen/PDMS and PDMS) and times of extraction (15, 30 and 45 min) were tested. For comparative purposes and validation of the SPME methodology, methanol and acetic acid contents in polysaccharide samples were also determined by direct injection of the aqueous solution in the GC–FID.

2. Materials and methods

2.1. Materials

Standards used were of analytical grade. Methanol (99.0%) and acetic acid (99.5%) were purchased from Fluka and Riedel, respectively. Calibration curves were made from readily prepared methanol (0.8 g L⁻¹) and acetic acid (1 g L⁻¹) stock solutions. A SPME holder from Supelco, Inc. (Bellefonte, PA, USA) was used to perform headspace-SPME manually. SPME fibres (Supelco, Inc., Bellefonte, PA, USA) coated with 50/30 µm divinylbenzene/Carboxen on polydimethylsiloxane (DVB/Carboxen/PDMS) and 100 µm polydimethylsiloxane (PDMS) were conditioned prior to use at 250 °C for 4 and 0.5 h, respectively, according to the manufacturer's recommendations.

2.2. Sample origin and preparation

Polysaccharides were obtained from the pulp washing water and from the alcohol insoluble residue (AIR) of plums (*Prunus domestica*, var. 'Rainha Claudia'). Pectic polysaccharide-rich samples were also obtained by extraction of the AIR with water

and 0.5 M imidazole/HCl pH 7.0, as described by Mafra et al. (2001). Each sample, 2 mg for water and imidazole extracts and 5 mg for AIR, was dispersed in water (2.4 mL) in vials with 10 mL capacity and sonicated for 10 min in a water bath at room temperature. The saponification of the polysaccharides occurred by the addition of 0.8 mL of 2 M NaOH, with a reaction time of 1 h at 25 °C, as described by Waldron and Selvendran (1990). The reaction was finished by the addition of 0.8 mL of 2 M HCl, and the pH was adjusted to 2.0.

2.3. Determination of methanol and acetic acid by HS-SPME–GC–FID

The vials (10 mL) containing 4.0 mL of sample suspension (sample dispersed in 2.4 mL of water, 0.8 mL of 2 M NaOH and 0.8 mL of 2 M HCl) or standard solutions were thermostated at 40 °C in a water bath, with continuous stirring. After 15 min, the SPME fibre was manually inserted through the Teflon septum into the headspace of the vial. Since headspace volume can be a critical factor determining the precision of the results in three-phase systems, vials from the same producer and lot were used.

For HS-SPME optimization, for both DVB/Carboxen/PDMS and PDMS fibre coatings, three times of extraction were used, 15, 30 and 45 min. The analysis of the samples was performed using the DVB/Carboxen/PDMS fibre coating exposed at 40 °C during 30 min.

The SPME coating fibre containing the headspace volatile compounds was introduced into the GC injection port at 250 °C and kept for 10 min for the desorption. A Hewlett-Packard 5890 series II gas chromatograph (Hewlett-Packard, Wilmington, USA), equipped with a split/splitless injector and a flame ionisation detector (FID) was used. The injection port was lined with a 0.75 mm i.d. splitless glass liner. Splitless injection mode was used (3 min). The desorbed compounds were separated in a 30 m length DB-Wax column (J&W) with 0.53 mm i.d. and 1.0 µm film thickness and hydrogen as carrier gas was used at 6 mL/min linear velocity. A oven temperature programme was done between 50 and 220 °C with three rates, 5 °C min⁻¹ until 65 °C, 20 °C min⁻¹ until 185 °C, and 35 °C min⁻¹ until 220 °C, and held 1 min at 220 °C. The detector temperature was at 250 °C.

External calibration curves were constructed exposing the fibre to a standard mixture of methanol and acetic acid, for concentrations between 40 and 100 mg L⁻¹ for methanol and between 25 and 105 mg L⁻¹ for acetic acid. These concentration ranges were chosen according to the minimum and maximum content of methanol and acetic acid expected to find in plant polysaccharides. Calibration curves of methanol were also constructed in the presence of two concentrations of acetic acid, 80 and 105 mg L⁻¹, and calibration curves of acetic acid were constructed in the presence of 60 and 100 mg L⁻¹ of methanol.

All measurements were made with at least three replicates and the reproducibility was expressed as relative standard deviation (RDS, %). Blanks were run in between each set of experiments.

2.4. Determination of methanol and acetic acid by direct injection of the aqueous solution in the GC-FID

For comparative purposes and validation of the SPME methodology, methanol and acetic acid were also determined by direct injection of the aqueous solution in the GC-FID. An internal standard, 0.2 mL of 1-propanol, was also added to the vial after saponification and before the total volume of 4.0 mL has been completed with distilled water. The sample suspension was filtered through a glass fibre filter (Whatman GF/C) and a nylon membrane filter NL16 0.2 μm (S&S, Germany). The filtrate was injected (0.5 μL) in a GC HP 5890 series II with a FID detector under the conditions described in 2.3. Calibration curves were constructed for final concentration from 20 to 500 mg L^{-1} for both analytes. Estimated concentrations were made by peak area comparisons with the area of the known concentration of 1-propanol. The samples were prepared in duplicate and each run in duplicate ($n=4$).

2.5. Carbohydrate analysis

Neutral sugars were obtained by sulfuric acid hydrolysis (Selvendran, March, & Ring, 1979) and analyzed after conversion to their alditol acetates by GC, using 2-deoxyglucose as internal standard (Coimbra, Delgadillo, Waldron, & Selvendran, 1996). A Carlo Erba 6000 GC apparatus with split injector and a FID detector was used, with a 30 m column DB-225 (J&W) with i.d. and film thickness of 0.25 mm and 0.15 μm , respectively. The oven temperature program used was: initial temperature 200 $^{\circ}\text{C}$, a rise in temperature at a rate of 40 $^{\circ}\text{C min}^{-1}$ until 220 $^{\circ}\text{C}$ and then 220 $^{\circ}\text{C}$ for 14 min, followed by a rate of 20 $^{\circ}\text{C min}^{-1}$ until 230 $^{\circ}\text{C}$ and maintain this temperature for 1 min. The injector and detector temperatures were, respectively, 220 and 230 $^{\circ}\text{C}$. The flow rate of the carrier gas (H_2) was set at 1 mL min^{-1} .

Uronic acids (UrAc) were quantified by the 3-phenylphenol colorimetric method, as described by Coimbra et al. (1996).

3. Results and discussion

3.1. Optimization of HS-SPME-GC-FID methodology

In this study, two types of SPME fibre coatings were compared for their ability to extract methanol and acetic acid from the headspace.

PDMS fibre is a liquid coating recommended by the manufacturer for absorption of low molecular weight and non-polar volatile compounds (M_w , 60–275 Da). However, as PDMS 100 μm is the thickest coat available for SPME, its thick polymer coat also allowed it to be successfully applied in the microextraction of polar volatile compounds (Kataoka et al., 2000; Mester et al., 2001). DVB/Carboxen/PDMS is a mixed coating fibre used for a wide range of analytes (volatile, semivolatile and polar compounds— C_{3-20} , M_w 40–275 Da). It is reported as most sensitive for small compounds and organic acids due to its increase retention capacity resultant from the mutually potentiating effect of adsorption and absorption to

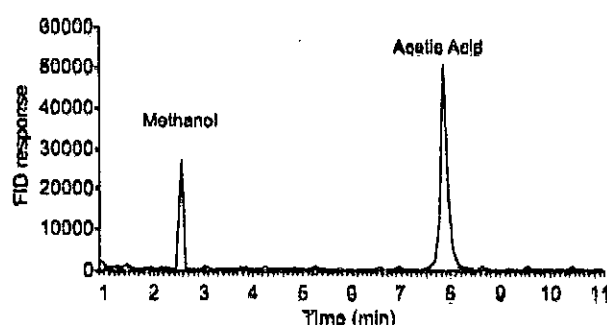


Fig. 1. HS-SPME-GC-FID chromatogram for separation of methanol (2.5 min) and acetic acid (7.8 min).

the stationary phase. The small pores (10 $\text{\AA}$ on average) of Carboxen make this coating fibre particularly effective for extracting small molecules (Kataoka et al., 2000).

In the case of the HS-SPME in a three phase system, the amount of an analyte sorbed on the fibre, and the resulting sensitivity, are determined both by sorption kinetics and by the distribution coefficient of the compound between the coating fibre, the headspace and the sample. SPME is, however, sensitive to experimental conditions. Any change in

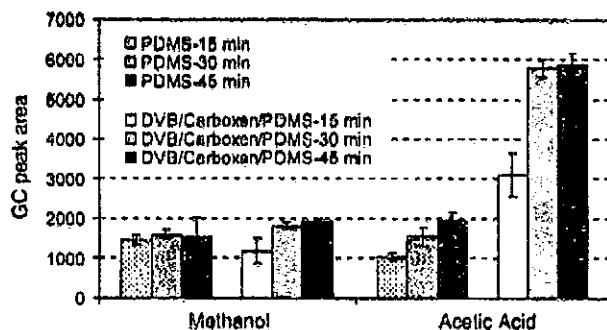


Fig. 2. GC peak areas for methanol and acetic acid using PDMS and DVB/Carboxen/PDMS fibre coatings at three different times of extraction. Solution contained 40 g L^{-1} methanol and 52 g L^{-1} acetic acid at pH 2.0.

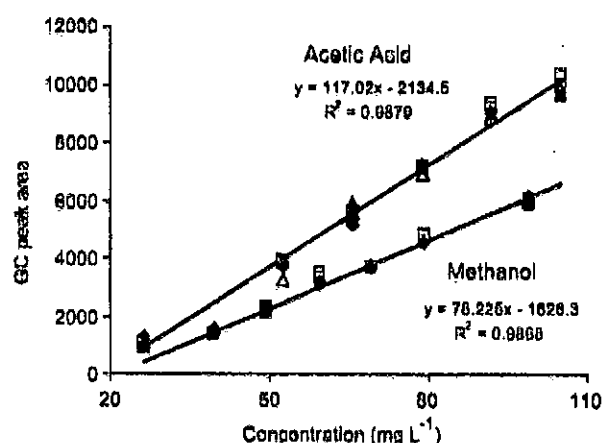


Fig. 3. Calibration curves for methanol with: no acetic acid ($\blacklozenge$), 80 mg L^{-1} acetic acid ($\square$), and for acetic acid with: no methanol ($\blacklozenge$), 60 mg L^{-1} methanol ($\square$) and 100 mg L^{-1} methanol ($\triangle$).

Table 1
Slope, intercept and *r*-squared values for calibration curves of methanol and acetic acid by HS-SPME-GC-FID

Calibration curves	Slope	Intercept	<i>r</i> -squared
<i>Methanol</i>			
0 µg mL ⁻¹ acetic acid	75.9 ± 1.6 ^a	-1449 ± 108 ^a	0.996
80 µg mL ⁻¹ acetic acid	76.5 ± 3.3 ^a	-1464 ± 228 ^a	0.981
105 µg mL ⁻¹ acetic acid	82.3 ± 2.5 ^a	-1964 ± 175 ^a	0.991
Total data for methanol	78.2 ± 1.6 ^b	-1626 ± 107 ^b	0.987
<i>Acetic acid</i>			
0 µg mL ⁻¹ methanol	114.9 ± 3.6 ^a	-1993 ± 265 ^a	0.991
60 µg mL ⁻¹ methanol	119.3 ± 3.3 ^a	-2161 ± 249 ^a	0.992
100 µg mL ⁻¹ methanol	116.7 ± 4.6 ^a	-2243 ± 342 ^a	0.985
Total data for acetic acid	117.0 ± 2.3 ^b	-2134 ± 167 ^b	0.988

^a Standard deviation (*n* = 18).

^b Standard deviation (*n* = 54).

experimental parameters, which affects the distribution coefficient and the sorption rate, will influence the amount sorbed and the corresponding reproducibility and sensitivity of the method (Yang & Peppard, 1994). Then, the time required for the extraction of the analytes from headspace was also evaluated for both coatings.

Independently of the type of fibre coating, time of extraction and concentration of analytes, high chromatographic resolution for methanol and acetic acid was observed in the 11 min temperature program. Methanol eluted near 2.5 min (65 °C) while acetic acid eluted near 7.8 min (165 °C), as is shown in Fig. 1. Fig. 2 shows the GC peak areas for methanol and acetic acid using both types of SPME coatings with different time of extraction.

For acetic acid the areas were three times higher using the DVB/Carboxen/PDMS fibre than the PDMS, while for methanol the areas obtained with the two coatings were similar. The reproducibility obtained for both analytes, shown by the error bars in Fig. 2, is better for times of extraction higher than 30 min when DVB/Carboxen/PDMS fibre was used, revealing that DVB/Carboxen/PDMS fibre is a better coating for methanol and acetic acid extraction than PDMS.

Using DVB/Carboxen/PDMS fibre, the areas for both analytes increased almost twice with an increase from 15 to 30 min of the extraction time, while no significant changes were observed with increasing the time of extraction to 45 min. These results allow to infer that the equilibrium of the amount of compounds in the sample and in the fibre coating was reached, at least, in 30 min. Therefore, 30 min of extraction at 40 °C with DVB/Carboxen/PDMS fibre coating was chosen as the optimal experimental conditions for the analysis of methanol and acetic acid by HS-SPME.

3.2. Calibration curves of methanol and acetic acid by HS-SPME-GC-FID

External calibration curves for quantification of methanol and acetic acid were performed adding the standards in the concentration range between 40 and 100 mg L⁻¹ for methanol and between 25 and 105 mg L⁻¹ for acetic acid. The concentrations used were those expected to occur in the polysaccharide samples. The solution matrix was chosen to have the same composition of those containing the saponified

Table 2
Sugars composition of polysaccharides samples from plums and methanol and acetic acid released from them (mg g⁻¹ of polysaccharide sample), determined by HS-SPME-GC-FID and by direct injection of the aqueous solution in the GC-FID

Polysaccharide sample	Sugars composition (mol%)								Total sugars (mg g ⁻¹)	HS-SPME-GC-FID ^a		Direct injection-GC-FID ^b	
	Rha	Fuc	Arn	Xyl	Man	Gal	Glc	UrAc		Methanol (mg g ⁻¹)	Acetic acid (mg g ⁻¹)	Methanol (mg g ⁻¹)	Acetic acid (mg g ⁻¹)
<i>Pulp water washing</i>													
PW	2	0	13	2	5	7	27	45	653	40.0 (3)	–	37.5 (7)	–
<i>AIR</i>													
AIR1	1	1	16	3	2	10	25	42	687	20.5 (6)	11.0 (11)	22.2 (8)	13.8 (10)
AIR2	2	1	14	4	3	12	31	32	618	21.8 (2)	13.1 (10)	22.4 (8)	15.4 (10)
AIR3	2	0	10	4	3	12	42	28	605	15.6 (7)	13.8 (10)	17.3 (7)	15.2 (10)
AIR4	2	1	14	5	2	17	20	41	818	27.5 (7)	7.3 (8)	28.0 (1)	9.3 (10)
<i>AIR water extract</i>													
W1	1	1	13	4	3	7	33	38	562	14.6 (6)	12.4 (5)	13.9 (6)	15.1 (11)
W2	1	0	5	2	5	3	67	15	635	9.2 (11)	10.0 (9)	8.5 (3)	10.3 (11)
W3	1	0	4	1	3	3	72	15	678	16.7 (8)	30.5 (7)	15.6 (4)	34.0 (8)
W4	2	1	10	2	1	10	6	69	997	92.0 (8)	16.7 (3)	87.9 (1)	19.5 (11)
<i>AIR imidazole extract</i>													
Im1	1	0	9	2	1	8	2	77	940	98.2 (2)	–	94.5 (6)	–
Im2	2	1	12	2	2	12	3	66	770	60.5 (5)	–	62.5 (7)	–
Im3	1	1	20	1	1	9	2	65	952	69.7 (1)	–	62.9 (9)	–
Im4	2	1	25	1	1	14	2	54	837	56.2 (7)	26.2 (8)	53.1 (2)	24.2 (10)

^a Numbers in parentheses correspond to the coefficient of variation (%) (*n* = 3).

^b Numbers in parentheses correspond to the coefficient of variation (%) (*n* = 4).

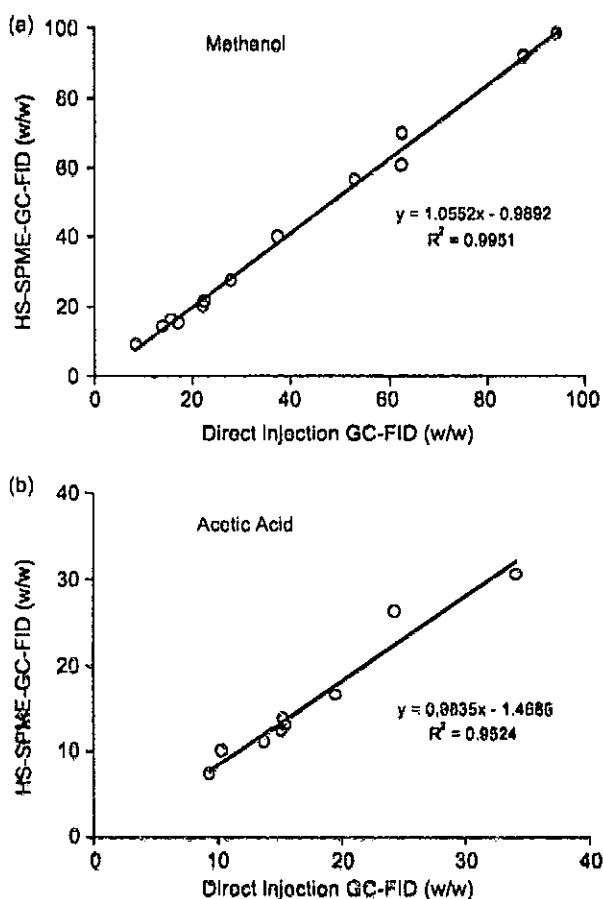


Fig. 4. Comparison of (a) methanol and (b) acetic acid contents (w/w) of polysaccharides, obtained by HS-SPME-GC-FID and by direct injection in GC-FID.

polysaccharide samples, that is, 0.8 mL of NaOH 2 M and 0.8 mL of HCl, pH 2, in a total volume of 4.0 mL.

SPME usually gives a linear response between the concentration of the analyte in solution and the amount of analyte extracted (Kataoka et al., 2000; Mester et al., 2001). However, for porous polymer fibres, like DVB/Carboxen/PDMS, the competitive adsorption process due to the limited surface area available for adsorption may result in the displacement of analytes with lower affinities, affecting therefore the estimation of their concentration (Górecki, Yu, & Pawliszyn, 1999; Mester et al., 2001; Pawliszyn, 2000). In order to evaluate the effect of a possible interference of the variation of the concentration of methanol on the amount estimated of acetic acid and the effect of variation of the concentration of acetic acid on the amount estimated of methanol, calibration curves of each analyte were also constructed in the presence of two different concentrations of the other, 80 and 105 mg L⁻¹ of acetic acid and 60 and 100 mg L⁻¹ of methanol (Fig. 3). The values of slope, intercept and *r*-squared for the six calibration curves are presented in Table 1. No statistical differences (*p* < 0.05) were observed among the three curves obtained for each compound in the presence of the other. These results allowed to conclude that during the extraction procedure with SPME, within this

concentration range, no significant competitive interferences occur between these compounds.

The calibration curves used to quantify the methanol and acetic acid content from polysaccharide extracts were obtained using all the values, nine determinations for each concentration (values showed in 'Total' line in Table 1). A linear relationship between the concentration of methanol and acetic acid and their GC peak area was observed, with a *r*-squared values of 0.988 for methanol and 0.987 for acetic acid (Table 1). Based on the standard deviations obtained, the reproducibility of this methodology, expressed as a percentage of the mean of the standard area for each concentration, was estimated to be below 10% for both analytes. Quantification limits, determined by the concentration of each standard required to give a peak height ten times higher than the noise, was estimated to be 32 mg L⁻¹ for methanol and 26 mg L⁻¹ for acetic acid.

3.3. Determination of methanol and acetic acid contents of polysaccharides

Methanol and acetic acid contents were determined for a set of plum polysaccharides, obtained from the pulp washing water, from the alcohol insoluble residue (AIR), and from extraction of the AIR with water and 0.5 M imidazole/HCl pH 7.0. These samples differ in their sugar composition (Table 2), namely uronic acids (15–77 mol%) and total amount of sugars (562–997 mg g⁻¹). Methanol and acetic acid content after saponification of samples were determined by HS-SPME-GC-FID and by direct injection of the aqueous solution in the GC-FID, to compare the results, since this methodology has been used for quantification of methanol and acetic acid content of polysaccharides. Statistical significant differences between the two methods for the values of methanol and acetic acid obtained for all polysaccharides analysed were evaluated using *t*-student test at the *p* < 0.05 level. No significant differences were observed. The two methodologies can also be compared by the linear relationship for the estimation of both methanol and acetic acid contents (Fig. 4). For methanol, the calculated linear regression had a slope of 1.05 ± 0.05 and a intercept of -2.31 ± 3.21, while for acetic acid the slope was 0.98 ± 0.04 and the intercept was -0.54 ± 0.55. These results show that the calculated slope and intercept for both regression trend lines do not differ significantly from 1 and 0, respectively, and thus there is no evidence for differences between the results obtained by both methods. The reproducibility of the proposed HS-SPME-GC-FID methodology, when applied to the samples, was also shown to be near 10%, a value similar to that found for the standards.

4. Concluding remarks

A simple, rapid and solvent-free methodology for simultaneous determination of methanol and acetic acid released by saponification from plant cell wall polysaccharides is proposed. This methodology comprises the study of the sample headspace by SPME using a DVB/Carboxen/PDMS coating

fibre followed by a GC-FID analysis. For quantitative purposes external calibration curves were used. The results demonstrate a methodology that could be used for analysis of plant cell wall polysaccharides independently of their origin and type, namely, the water insoluble and cellulose-rich AIRs, the highly methylesterified and acetylated pectic polysaccharides of the imidazole extracts or the unpurified water soluble glucan and pectic polysaccharides.

This is a much easier and less time consuming methodology for sample preparation than titration, enzymatic or chemical methods and allows the simultaneous determination of methanol and acetic acid. It is cleaner than the chromatographic methods that require direct injection of acidic aqueous sample solutions. This methodology does not require the use of isotope internal calibration and mass spectrometry, not always available as a laboratory facility. HS-SPME-CG-FID methodology provides a simple, solvent-free, and reproducible procedure, compared to the already established methodologies used for methanol and acetic acid estimation.

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