Medellín, 26 de septiembre de 2019 **[M.DIE-528-19]**

Señores
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES
E. S. D.

Re.: Expediente No. NC2016/0005759
Solicitud de patente de invención titulada:
"PROCESO PARA OBTENER UN EXTRACTO BIOCIDA"
Solicitante: UNIVERSIDAD NACIONAL DE COLOMBIA

RESPUESTA AL OFICIO No. 5795, NOTIFICADO EL 05 DE JULIO DE 2019

(PRIMER EXAMEN DE FONDO)

PATRICIA ARROYAVE FRANCO, identificada como aparece al pie de mi firma, en mi condición de apoderada de la sociedad solicitante, me permito manifestar lo siguiente ante el auto emanado por ese Despacho, dentro del plazo legal previsto en el artículo 45 de la Decisión 486, en los siguientes términos:

1. En primer lugar, con el ánimo de brindar mayor claridad a la materia reivindicada, me permito presentar un **Capítulo Reivindicatorio Modificado**, que reemplaza en su totalidad al Capítulo Reivindicatorio presentado originalmente.

Así entonces, en el Capítulo Reivindicatorio Modificado se introdujeron los siguientes cambios:

- se eliminaron las Reivindicaciones 2, 4 y 8;
- en la Reivindicación 1, inciso (a), se introdujeron las limitaciones de la antigua Reivindicación 2 y, en el inciso (c), se introducen las limitaciones de la antigua Reivindicación 4;

- las nuevas Reivindicaciones 2 a 5 corresponden a las antiguas Reivindicaciones 3 y 5 a 7, respectivamente.

Valga anotar que las modificaciones efectuadas en el Capítulo Reivindicatorio cumplen a cabalidad con lo estipulado en el Artículo 34 de la Decisión 486, ya que estas de ninguna manera implican una ampliación de la protección correspondiente a la divulgación contenida en la solicitud original. Por el contrario, estas corresponden a una drástica limitación de la materia reclamada originalmente.

2. a- El Despacho eleva las siguientes objeciones en relación con el Capítulo Reivindicatorio:

- la Reivindicación 1 carece de claridad y concisión puesto que no especifica las condiciones ambientales necesarias para incubar el pre-inóculo con la resina adsorbente hidrofóbica y el tipo de resina utilizada. Además, dicha reivindicación menciona de manera general un "microorganismo" y una "resina adsorbente hidrofóbica", que corresponde a un conjunto indeterminado de posibles sustancias, de tal manera que no es posible determinar el alcance de lo que se desea proteger. El Despacho sugiere complementar la reivindicación con dichas condiciones ambientales, el tipo de microorganismo y la resina adsorbente hidrofóbica;
- la Reivindicación 5 carece de concisión porque describe una "resina adsorbente de tipo polimérico" la cual corresponde a un conjunto indeterminado de posibles sustancias;
- la Reivindicación 7 no es clara, porque caracteriza el extracto biocida (un producto) en función del proceso de obtención, lo cual no corresponde a una característica técnica para este producto;
- la Reivindicación 8 no es patentable porque reclama un uso.

b. i) En respuesta a las objeciones del Despacho, en primer lugar me permito llamar respetuosamente la atención del mismo sobre las modificaciones realizadas en el Capítulo Reivindicatorio Modificado adjunto, como lo indiqué en el numeral 1 del presente memorial.

En particular, se eliminó la Reivindicación 8 por lo que considero completamente superada la objeción relacionada con materia no patentable.

ii) Adicionalmente, con el ánimo de facilitar el trámite y siguiendo las amables sugerencias del Despacho, la Reivindicación 1 se modificó de acuerdo con las antiguas Reivindicaciones 2 y 4 definiendo así tanto el término "microrganismo" como las condiciones ambientales de la etapa (c). Es decir, en el inciso (a) de la Reivindicación 1 se restringió el término "microorganismo" a aquellos del género *Bacillus* sp*, Serratia* sp*, Pseudomonas* sp*, Burkholderia* sp*, Brevundimonas* sp*, Escherichia* sp*, Delftia* sp*, Acinetobacter* sp*, Photorhabdus* sp o *Xenorhabdus* sp (antigua Reivindicación 2); y, a su vez, en el inciso (c) se restringieron las condiciones ambientales de temperatura, tiempo, pH y agitación (antigua Reivindicación 4).

Así, considero respetuosamente que el proceso reclamado en la Reivindicación 1 se encuentra plenamente definido por sus etapas y condiciones esenciales de tal forma que ya no se reclama un "conjunto indeterminado" sino que por el contrario se reclaman características definidas que resultan completamente claras y concisas para cualquier persona medianamente versada en la materia, haciendo que el alcance de la protección resulte claro y conciso, y evitando cualquier ambigüedad. Por lo tanto, respetuosamente considero completamente superadas las objeciones al respecto al tipo de microorganismo y condiciones ambientales.

iii) Ahora bien, en relación con la supuesta falta de concisión del Capítulo Reivindicatorio debido al uso de las expresiones *"resina adsorbente hidrofóbica"* y "*resina adsorbente de tipo polimérico*" actualmente incluida en las nuevas Reivindicaciones 1 y 3, respectivamente, me permito señalarle respetuosamente al Despecho que de acuerdo con la

Guía para Examen de Solicitudes de Patente de Invención y Modelo de Utilidad de la Superintendencia de Industria y Comercio (en adelante, Guía de la SIC) el significado y alcance de los términos de las reivindicaciones debe ser el que normalmente se les da en el área técnica de la solicitud, y tiene que ser claro para la persona medianamente versada en la materia con la sola lectura de las reivindicaciones, si el término tiene un significado **especial** dado por la definición en el Capítulo Descriptivo, esta definición deberá incluirse en la reivindicación.[1] En el presente caso, las expresiones *"resina adsorbente hidrofóbica"* y "*resina adsorbente de tipo polimérico*" no tienen un significado diferente o especial frente a lo que se conoce en el área técnica de la química o ingeniería química.

Tan es así que en la información básica pública gratuita a la que tiene acceso cualquier persona desde internet se define la extracción en fase sólida como "*un método en el que se unen grupos funcionales hidrofóbicos a superficies de partículas sólidas y funcionan como fase extractora*".[2] De allí se entiende, al menos como regla general elemental, que cualquier proceso de extracción en fase sólida involucrará inequívocamente la unión de grupos funcionales **hidrofóbicos** (es decir, resina adsorbente hidrofóbica) con superficies sólidas, tal como se reclama en la presente invención.

No obstante la anterior, me permito aportar ocho documentos que hacen parte del Estado general del arte (ANEXOS 1 a 8) y que demuestran que este tipo de resinas son usadas en extracción en fase sólida, y por lo tanto, sus características estructurales son entendidas por cualquier persona medianamente versada en la materia.

Por ejemplo, en el documento "*A guide to solid phase extraction*"[3] (ANEXO 1) se señala en la página 117 que: "*Depending on the monomers used to build the **polymeric resin**, and the processes used during polymerisation, many resins can be used for solid phase extraction without further processing. These resins **are hydrophobic** and offer a single, extremely non-*

[1] Superintendencia de Industria y Comercio (2014). Guía para Examen de Solicitudes de Patentes de Invención y Modelo de Utilidad. (2014), Sección 2.6.5.1.
[2] Definición Extracción Fase Sólida: https://es.wikipedia.org/wiki/Extracci%C3%B3n_en_fase_s%C3%B3lida
[3] A guide to solid phase extraction. International Sorbent Technology (IST). Recuperado de http://www.westernanalytical.com/pdf/Help_58.pdf

selective retention mechanism […]". *"In general,* ***all resins*** *have significant* ***hydrophobic*** *character due to the nature of the* ***polymer*** *backbone, whereas the hydrophobic nature of silica based sorbents is entirely dependant on the bonded groups […]".*

Por su parte, en el documento "*Assessing the effects of adsorptive polymeric resin additions on fungal secondary metabolite chemical diversity*"[4] (ANEXO 2) en la página 179 se menciona que "***Adsorptive polymeric resins*** *have been incorporated since 1970s into fermentation processes for myxobacteria* […]"

En el documento "*Overview of the methods for purification of metabolites that are secreted by bacteria*"[5] (ANEXO 3) se establece que "*An example of an* ***adsorbent*** *that may be used for this method is beads of Amberlite XAD-2 from MilliporeSigma. This material is a* ***hydrophobic copolymer*** *of styrene-divinylbenzene* ***resin***".

A su vez en el documento correspondiente a la ficha técnica comercial del producto AMBERLITE XAD16[6] (ANEXO 4) en el primer párrafo se señala que "*AMBERLITE XAD16 is a* ***polymeric*** ***adsorbent*** *supplied as insoluble white beads. It is a nonionic,* ***hydrophobic, crosslinked polymer*** *which derives its* ***adsorptive properties*** *from its patented macroreticular structure [...]*".

En el ANEXO 5, document titulado "*Solid-Phase Extraction Comparison of (SPE)—a 16 Materials for the Purification of Anthocyanins from Aronia melanocarpa var Nero*"[7], se señala en el abstract que "*Among these, reversed-phase silica gels and macro-reticular non-*

[4] González-Menéndez, V. et al. (2014). Assessing the effects of adsorptive polymeric resin additions on fungal secondary metabolite chemical diversity. Mycology. Vol. 5, No. 3, 179–191, http://dx.doi.org/10.1080/21501203.2014.942406

[5] Crowley, T. (2020). Purification and Characterization of Secondary Metabolites. Chapter 3: Overview of the methods for purification of metabolites that are secreted by bacteria. Academic Press. https://doi.org/10.1016/C2017-0-00857-1

[6] AMBERLITE® XAD16 Industrial Grade Polymeric Adsorbent. Product Data Sheet. (2003). Rohm and Haas Company.

[7] Kraemer-Schafhalter, A. (1998). Solid-Phase Extraction Comparison of (SPE)—a 16 Materials for the Purification of Anthocyanins from Aronia melanocarpa var Nero. J Sci Food Agric, 78, pp. 435-440

*ionic **acrylic polymer adsorbents** such as Serdolit PAD IV or **Amberlite XAD-7** turned out to be most suitable*.".

De forma similar, en el ANEXO 6 en la ficha técnico-comercial para las resinas de adsorción "AMBERLITE XAD POLYMERIC RESINS Sigma Prod. Nos. XAD-4, XAD-7, XAD-16, 1-0377,1-0393, y 1-0379"[8] se señala en la tabla de la última página que para los siete tipos de resinas ofrecidas la NATURALEZA QUÍMICA de todas ellas es "***Hydrophobic poly**aromatic*".

Finalmente, el ANEXO 7 "*Chemically modified polymeric resins for solid- phase extraction and group separation prior to analysis by liquid or gas chromatography*"[9] y el ANEXO 8 "*Principles and practice of solid-phase extraction*"[10] son documentos que hablan de la generalidad del arte en materia de extracción en fase sólida y además indican que usar resinas poliméricas es uno de los elementos esenciales del proceso de extracción en fase sólida y que estas son preferidas por encima de las resinas de silica.

Así, de lo anterior se desprenden dos ideas: la primera, que por practicidad los investigadores y comercializadores de resinas se refieren en sus estudios, fichas técnicas e información técnico-comercial a las marcas comerciales para identificar las resinas y materiales adsorbentes utilizados/comercializados; y segundo que las resinas usadas en extracción en fase sólida son principalmente adsorbentes, hidrofóbicas y de carácter polimérico tal como se reclaman en la presente invención.

Respecto a la primera idea, valga la pena resaltar que en materia de patentes no es posible reclamar las resinas por su marca comercial como si se puede hacer en artículos científicos o información técnico-comercial. Tan es así que el Manual Andino de Patentes en la sección

[8] Amberlite® XAD16N. Product Information Sheet. Recuperado de: https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Product_Information_Sheet/xad16pis.pdf

[9] Schmidt, Luther W., "Chemically modified polymeric resins for solid-phase extraction and group separation prior to analysis by liquid or gas chromatography" (1993). Retrospective Theses and Dissertations. 10268.

[10] Pawliszyn, J (2002). Sampling and Sample Preparation for Field and Laboratory. Poole, C. Chapter 12: Sampling and Sample Preparation for Field and Laboratory. Elsevier.

4.6.4 señala que "*Las marcas registradas o nombres comerciales definen productos o proceso que pueden cambiar con el paso del tiempo aunque conserven el mismo nombre. Por ello su uso en una reivindicación no está permitido ya que no permiten establecer el alcance de la reivindicación*".[11] Por esta razón, el solicitante debe recurrir obligatoriamente a expresiones que definan en términos generales la naturaleza química de los materiales utilizados en el desarrollo del concepto inventivo. De esta manera el Despacho no puede confundir que el Solicitante se vea en la obligación de definir los materiales utilizados mediante los nombres o expresiones genéricas más próximas a las características estructurales esenciales del material reclamado buscando delimitar el alcance con que paradójicamente estas expresiones representen *per se* un concepto amplio, vago e indeterminado como equivocadamente ocurre en el presente caso.

Es importante indicar que las características esenciales estructurales que se reclaman en la presente invención para la resina es que sea, precisamente, una resina adsorbente hidrofóbica y una resina adsorbente de tipo polimérico De esta forma, es evidente que dichas expresiones son claras y comprensibles para cualquier persona medianamente versada en la materia puesto que sin necesidad de acudir a información adicional están en la capacidad de informar exactamente a que componente o estructura se está haciendo referencia cuando se reclama una resina adsorbente hidrofóbica y una resina adsorbente de tipo polimérico.

Adicionalmente, respetuosamente le recuerdo a la Oficina que las reivindicaciones deben proteger el concepto inventivo y no restringirse a modalidades específicas, de tal manera que la definición de dichas expresiones componentes químicos o físicos específicos limitaría injustamente la materia reclamada en tanto lo que hace inventivo al proceso de la Reivindicación 1 son las etapas y condiciones reclamadas, y no las expresiones objetadas.

Por tanto, me permito resaltar que un inventor está en su derecho de reclamar protección para la totalidad del concepto inventivo desarrollado, **sin importar la amplitud de éste**, siempre y cuando se conserve el principio de unidad de invención y no haya arte previo que afecte su

[11] Comunidad Andina de Naciones (2004). *Manual Andino de Patentes*. Sección 4.6.4, p. 40

patentabilidad. Es decir, una reivindicación puede ser tan amplia como se requiera para así poder enmarcar la totalidad del concepto inventivo desarrollado y dicha amplitud no necesariamente implica falta de claridad. En este caso las expresiones *"resina adsorbente hidrofóbica"* y "*resina adsorbente de tipo polimérico*" son completamente claras para la persona medianamente versada en la materia en tanto corresponden al lenguaje del área técnica a la cual pertenece la presente invención, como acaba de demostrarse anteriormente.

En este punto resulta relevante recordar que el Capítulo Descriptivo de una solicitud de patente está dirigido a personas medianamente versadas en la materia, esto es, personas en el área de la química las cuales están en total capacidad de comprender que tales expresiones son alusivas a ciertas modalidades de la invención las cuales simplemente representan diferentes materializaciones que conforman las etapas del proceso reclamado, los cuales son comúnmente empleados en la técnica, como insisto, fue sustentando anteriormente con la bibliografía aportada.

Así, en el presente caso, la materia reclamada en el Capítulo Reivindicatorio Modificado adjunto corresponde al concepto inventivo desarrollado por el inventor, y no debería existir obligación alguna de limitar a componentes químicos específicos de la resina, pues esta medida reduciría injustamente el concepto inventivo a que el inventor tiene derecho. Por lo tanto, no existe requerimiento alguno que determine que los ejemplos deban ilustrar todas y cada una de las materializaciones de cada una de las etapas que se encuentran dentro del alcance de la invención, o que las reivindicaciones sólo pueden reclamar lo ilustrado en los ejemplos, ya que lo fundamental es que el Capítulo Descriptivo proporcione la información necesaria para que la persona medianamente versada en la materia pueda reproducir la invención, sin tener que acudir a experimentación adicional excesiva. Además, vale la pena recordar que la función de los Ejemplos en el Capítulo Descriptivo es **estrictamente ilustrativa** y de ninguna manera puede considerarse como el único alcance reivindicable posible. De hecho, reclamar estrictamente aquello ejemplificado en el Capítulo Descriptivo limita injustamente el alcance de la protección a la cual el inventor tiene derecho, de tal forma que para que existiera una infracción, dichos elementos específicos ilustrados en los ejemplos

deberían necesariamente estar presentes en el producto o proceso infractor, lo que fácilmente podría lograrse al tratarse de elementos que pueden ser cambiados o modificados.

Por consiguiente, para el presente caso, la persona medianamente versada en la materia se encuentra en total capacidad de comprender el alcance de la invención, pues notaría que éste se encuentra dentro de la materia divulgada en el Capítulo Descriptivo y sería capaz de reproducir la materia reclamada sin acudir a experimentación adicional excesiva.

De esta manera, dichas expresiones objetadas por el Despacho son claras, concisas y además son conocidas por cualquier persona medianamente versada en la materia dentro del lenguaje técnico usual y estándar dentro del campo de la tecnología de la extracción en fase sólida por lo que considero superada cualquier objeción al respecto.

iv) En cuanto al extracto biocida de la Reivindicación 4 (antigua Reivindicación 7), respetuosamente difiero de la opinión del Despacho puesto que en este caso se cumplen los requisitos establecidos por el Manual Andino de Patentes (MAP) para la aceptación de las reivindicaciones de producto por proceso. En particular, el MAP señala que "*las reivindicaciones de productos definidas en términos de un proceso de fabricación son admisibles si los productos como tales cumplen los requisitos de patentabilidad, […] y cuando no se pueden definir por sus características estructurales"*[12]. Con base en lo anterior, en el presente caso, el producto resultante del método reclamado (el extracto) no puede ser definido mediante sus características estructurales dada su ineludible naturaleza química de convivencia simultánea y entrelazada de moléculas lo cual hace imposible su elucidación estructural determinada. Por esta razón considero que la nueva Reivindicación 5 cumple con los requisitos técnicos de tal forma que los límites de protección reclamados resultan claros y concisos para cualquier persona medianamente versada en la materia por lo que debe ser aceptada.

[12] Comunidad Andina de Naciones (2004). *Manual Andino de Patentes*. Sección 4.6.8, p. 41

v) En conclusión, considero que las objeciones elevadas en el examen de fondo con respecto a falta de claridad y concisión del Capítulo Reivindicatorio Modificado quedan superadas, por lo que éste cumple con lo establecido en el artículo 30 de la Decisión 486, así como aquello dispuesto por el Manual Andino de Patentes (MAP) y la Guía de la SIC.

3. a- El Despacho sugiere adicionar en las Tablas 6 a la 20 del Capítulo Descriptivo un título que contenga una breve descripción del contenido e incluirlas en los resultados obtenidos. Adicionalmente señala que existe una inconsistencia en los números de referencia de las Tablas 7-20.

b- Considero superada la anterior objeción en tanto adjunto al presente memorial se encuentra un nuevo Capítulo Descriptivo en el cual se corrigen y subsanan las deficiencias sustanciales y formales (principalmente tipográficas) identificadas por el Despacho.

4. a- El Despacho argumenta que la invención carece de unidad de invención, debido a que reclama más de un grupo inventivo sin la presencia de características o elementos técnicos nuevos e inventivos. En especial, el Despacho sostiene que el concepto común hace referencia a un proceso para obtener un extracto biocida y se encuentra divulgado en el estado de la técnica, específicamente en el documento US2016/0073642 y US3817835 y el segundo grupo hace referencia a un extracto biocida obtenido a partir de diferentes microorganismos y se encuentra divulgado en el estado de la técnica, específicamente en los documentos: LI, X. et al. (2016), Jeong, H.U. (2010), Mavrodi, G. V., et al. (2012), Quan, C.S. et al. (2006), Al Attar, N. S. et al. (2015), Han, J. et al.(2005), San-Blas, E. et al. (2012), que son comunes a todas las invenciones.

Así, el Despacho identifica dos grupos inventivos, así:

- Grupo inventivo 1: Proceso para la obtención de un extracto biocida (reivindicaciones 1-2, 4 a 6).
- Grupo inventivo 2: Extracto biocida. En donde el grupo 2 puede ser subdividido en:

a) Extracto biocida obtenido a partir del microorganismo del género *Bacillus* sp.
b) . Extracto biocida obtenido a partir del microorganismo del género *Serratia* sp.
c) Extracto biocida obtenido a partir del microorganismo del género *Pseudomonas* sp.
d) . Extracto biocida obtenido a partir del microorganismo del género *Burkholderia* sp.
e) Extracto biocida obtenido a partir del microorganismo del género *Brevundimonas* sp.
f) Extracto biocida obtenido a partir del microorganismo del género *Escherichia* sp.
g) Extracto biocida obtenido a partir del microorganismo del género Delftia sp.
h) Extracto biocida obtenido a partir del microorganismo del género *Acinetobacter* sp.
i) Extracto biocida obtenido a partir del microorganismo del género *Photorhabdus* sp.
j) Extracto biocida obtenido a partir del microorganismo del género *Xenorhabdus* sp.

El Despacho sugiere restringir la materia a patentar en un solo concepto común inventivo especificando el avance tecnológico sobre el estado de la técnica.

b- En respuesta a esta objeción, con el ánimo de facilitar el trámite y siguiendo las amables sugerencias del Despacho, el solicitante decidió limitar el alcance de la Reivindicación 1 para especificar el microorganismo que se utiliza en el proceso reclamado. Así, en el inciso (a) el género del microorganismo exclusivamente a *Bacillus* sp, *Serratia* sp, *Pseudomonas* sp, *Burkholderia* sp, *Brevundimonas* sp, *Escherichia* sp, *Delftia* sp, *Acinetobacter* sp, *Photorhabdus* sp o *Xenorhabdus* sp.

De esta manera, la nueva Reivindicación 1 se encuentra dirigida a un único concepto inventivo que corresponde a un proceso que utiliza los microorganismos identificados por el Despacho en el grupo inventivo 2, y que tiene como resultado un extracto de cada uno de los microorganismos que se incorporan al método. Lo anterior no implica *per se* que se trate de invenciones diferentes sino que, por el contrario y como se infiere de la objeción y recomendación del Despacho, los microorganismos de grupo inventivo 2 (ahora incorporados en la Reivindicación 1 corresponden) a un proceso con 4 etapas que ocurren en cada uno de dichos microorganismos. Así, a pesar de reclamar en el inciso a) microrganismos diferentes, comparten las mismas características estructurales esenciales en las etapas y condiciones reclamadas en los incisos b), c) y d) convergiendo así en el mismo concepto inventivo desarrollado (unidad de materia).

Así, es importante mencionar las disposiciones de la Decisión 486, Artículo 25 en donde señala que la solicitud de patente solo podrá comprender una invención o **un grupo de invenciones relacionadas entre sí**, de manera que conformen **un único concepto inventivo**. Del mismo modo, el Manual Andino de Patentes en la sección 8.1 señala con respecto a la unidad de invención que cuando se trate de un **grupo de invenciones relacionadas entre sí por un único concepto inventivo** se debe considerar que el concepto inventivo único que relaciona las invenciones debe ser técnico, cumplir por sí mismo los requisitos de novedad y nivel inventivo y ser común a todas las reivindicaciones[13]. En el presente caso y como se sustentó anteriormente, las etapas y condiciones esenciales reclamadas en los incisos a), b), c) y d) corresponden a un concepto inventivo común que cumple los requisitos de novedad y además son comunes a todas las reivindicaciones por lo que el requisito de unidad de invención de la presente solicitud debería ser reconocido.

5. a- El Despacho señala que las Reivindicaciones 1, 2, 5 y 6 carecen de novedad a la luz de la patente US2016/0073642 (D1).

[13] Comunidad Andina de Naciones (2004). *Manual Andino de Patentes*. Sección 8.1, p. 60

El Despacho señala que D1 divulga un procedimiento para incrementar la producción de biomasa y metabolitos de especies de *Bacillus* sp, el cual comprende el cultivo del microorganismo en un medio de cultivo bajo condiciones ambientales específicas.

En particular, el Despacho presenta la siguiente tabla comparativa entre las características esenciales de la invención y las divulgadas en D1, así:

Tabla 1. Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1
Un proceso para obtener un extracto biocida que comprende las siguientes etapas:	Un procedimiento para incrementar la producción de biomasa y metabolitos de especies de *Bacillus* sp, que comprende las siguientes etapas: (Pág 10, reiv. 1)
a) aislar, purificar y cultivar un microorganismo;	Crecimiento del microorganismo en un medio de cultivo (Pág 10, reiv. 1, 4 y 5)
b) preparar un pre-inóculo del microorganismo;	Crecimiento del microorganismo en un medio de cultivo (Pág 10, reiv. 1, 4 y 5)
c) inocular el pre-inóculo con una resina adsorbente hidrofóbica bajo condiciones ambientales adecuadas; y	La biomasa obtenida a partir del proceso se incuba bajo unas condiciones ambientales específicas de temperatura, pH, agitación, aireación (Pág 10, reiv. 6 y 7) y es separada del medio de cultivo mediante centrifugación o microfiltración (Pág. 2, párr. [0037], pág. 10, reiv. 8).
d) separar y eluir la resina con un solvente orgánico hasta obtener el extracto bacteriano.	Los metabolitos son extraídos utilizando un solvente orgánico (Pág 10, reiv. 9).

De acuerdo con la tabla anterior, el Despacho sostiene que las características esenciales del proceso para obtener un biocida descrito en la reivindicación 1 habían sido divulgadas previamente en D1, por lo tanto, la materia reivindicada no es nueva.

Adicionalmente, D1 menciona la obtención de la biomasa a partir de especies de Bacillus sp., utilizando las siguientes condiciones ambientales: temperatura 20 - 40 °C, tiempo 10-100 horas, pH 5.5 a 7.5 y agitación de 400 a 600 rpm. Además de, un proceso de extracción utilizando metanol como solvente orgánico. Las reivindicaciones dependientes 2, 4 y 6 tampoco cumplen con el requisito de novedad.

b- i) En primer lugar, me permito llamar nuevamente la atención de la Oficina sobre las modificaciones realizadas en el Capítulo Reivindicatorio Modificado adjunto, en donde la Reivindicación 1 se modificó de tal manera que ahora incluye el alcance de las antiguas Reivindicaciones 2 y 4 limitando así tanto el tipo de microrganismo como las

condiciones ambientales de la etapa (c). Es decir, en el inciso (a) se restringió el género del microorganismo exclusivamente a *Bacillus* sp, *Serratia* sp, *Pseudomonas* sp, *Burkholderia* sp, *Brevundimonas* sp, *Escherichia* sp, *Delftia* sp, *Acinetobacter* sp, *Photorhabdus* sp o *Xenorhabdus* sp (antigua Reivindicación 2); y a su vez, en el inciso (c) se restringieron las condiciones ambientales esenciales de temperatura, tiempo, agitación y pH (antigua Reivindicación 4).

Por su parte, D1 divulga un proceso para incrementar la producción de biomasa y metabolitos de especies de *Bacillus*, donde dicho proceso incluye un medio de cultivo y condiciones específicas ambientales para la producción de grandes cantidades de biomasa y metabolitos de las familias fengicina, surfactina e iturina que tienen actividad antifúngica y antibacteriana contra varios patógenos. Particularmente, D1 describe un proceso para incrementar la producción de biomasa de microorganismos del género *Bacillus*, entre ellos *Bacillus subtilis y Bacillus amyloliquefaciens*, así como la de sus metabolitos biológicamente activos (lipopéptidos de la familia de las surfactinas, iturinas y fengicinas). Los metabolitos activos se extraen mediante procedimientos de extracción con solventes, precipitación, adsorción o cromatografía. Por el contrario, la presente invención se refiere a un proceso para obtener extractos biocidas a partir de microorganismos de diversos géneros entre los que se encuentran *Bacillus* sp, *Serratia* sp, *Pseudomonas* sp, *Burkholderia* sp, *Brevundimonas* sp, *Escherichia* sp, *Delftia* sp, *Acinetobacter* sp, *Photorhabdus* sp y *Xenorhabdus* sp. Los extractos obtenidos exhiben actividad fungicida, insecticida y/o de repelencia, en donde además se incluye el aislamiento y cultivo de los microorganismos.

A su vez, D1 divulga que se realiza un proceso de extracción con metanol de los metabolitos activos de los cultivos obtenidos de *B. subtilis* EA-CB0015 y *B. amyloliquefaciens* EA-CB0959. Posteriormente, se hace una extracción en fase sólida con metanol y las fracciones activas se purifican por HPLC de fase reversa con un detector UV con longitud de onda de 214 nm. Este proceso se realiza con el fin de obtener las fracciones activas y las moléculas responsables de la actividad, el producto se realiza con cultivo bacteriano (contiene células de la bacteria *B. subtilis*). En contraste, en la presente invención se inocula el pre-inóculo

con una resina adsorbente hidrofóbica bajo condiciones ambientales de temperatura, tiempo, pH y agitación en los siguientes rangos:

Condición	**Rango**
Temperatura (°C)	20 a 35
Tiempo (horas)	120 a 240
pH	5,5 a 8,0
Agitación (rpm)	100 a 200

Con base en lo anterior, es claro que D1 **no divulga** particular y explícitamente todas y cada una de las características estructurales del proceso reclamado en la presente invención. D1 no divulga explícitamente cada una de las etapas allí reclamadas, y, en particular, no divulga una etapa de inocular el pre-inóculo con una resina adsorbente hidrofóbica ni utiliza específicamente los microrganismos aquí reclamados. Nada en D1 divulga inequívocamente un proceso para obtener un extracto biocida que comprende las etapas de (a) aislar, purificar y cultivar un microorganismo, en donde dicho microorganismo es del género *Bacillus* sp, *Serratia* sp, *Pseudomonas* sp, *Burkholderia* sp, *Brevundimonas* sp, *Escherichia* sp, *Delftia* sp, *Acinetobacter* sp, *Photorhabdus* sp o *Xenorhabdus* sp; b) preparar un pre-inóculo del microorganismo; c) inocular el pre-inóculo con una resina adsorbente hidrofóbica bajo condiciones ambientales de temperatura, tiempo, pH y agitación mencionadas anteriormente, ni d) separar y eluir la resina con un solvente orgánico hasta obtener el extracto bacteriano, tal como se reclama en la presente invención. Es decir, existen diferencias estructurales entre las características esenciales aquí reclamadas y las divulgadas en D1.

A la luz de lo anterior, me permito resaltar que, para fundamentar una afectación válida contra la novedad de una invención, el MAP exige que el arte previo debe divulgar **explícitamente** todas y cada una de las características de la invención.[14] Adicionalmente, el Tribunal Andino de Justicia (TAJ) ha determinado que para establecer una objeción de novedad válida, la "*divulgación debe ser detallada y, en todo caso, suficiente para que una persona del oficio*

[14] Ibíde Comunidad Andina de Naciones (2004). *Manual Andino de Patentes* m, Sección 9.4

pueda utilizar esa información para ejecutar o explotar la invención".[15] En el presente caso, el documento citado no divulga todas las características del proceso aquí reclamado, en particular los microorganismos reclamados ni una etapa de extracción mediante una resina adsorbente hidrofóbica, por lo que su novedad debe ser reconocida.

6. Adicionalmente, por mera liberalidad del solicitante en aras de brindar mayor información, argumentos y evidencia en favor de los requisitos de patentabilidad de la presente invención frente al arte previo citado y con el propósito de facilitar el trámite, me permito llamar la atención sobre algunas otras diferencias que existen entre D1 y la presente invención, no solo a nivel estructural sino también funcional, en el sentido de demostrar la no obviedad del invento frente a las enseñanzas de D1, así:

a- Respecto al medio de cultivo, en D1 el medio de cultivo utilizado es el medio D (w/v): 3.34% de glucosa, 3.25% de extracto de levadura, entre 4.2x10% de sulfato de manganeso, 3.1x10% de Cloruro de calcio, 0.1% de sulfato de amonio, 0.4% de sulfato de magnesio, 0.1% de fosfato disódico y 0.05% de fosfato monosódico de potasio. Por el contrario, en la presente invención Los medios utilizados son: Medio XG y Medio NBTA: para aislamiento y cultivo. Medio nutritivo: extracto de carne 1,0 g, extracto de levadura 2,0 g, peptona 5,0 g y cloruro de sodio 5,0 g.

b- En cuanto a las condiciones de cultivo, referidas al aislamiento, purificación y cultivo, en la presente invención para el aislamiento, se colectan individuos de la hormiga *Atta cephalotes*, se maceran en un tubo de ensayo conteniendo esferas de vidrio y agua peptonada, se agitan a 2500-5000 rpm, se realizan diluciones seriadas entre 10^{-1} y 10^{-8}, se siembran en cajas de Petri con medio de cultivo y se incuban a una temperatura entre 20 y 35°C. El medio de cultivo debe contener fuentes de carbono y nitrógeno o los nutrientes necesarios para el crecimiento del microorganismo. En una modalidad preferida, el medio de cultivo comprende al menos una fuente de carbono, una fuente de nitrógeno, sales, buffers y nutrientes. Para el aislamiento de los microorganismos (v.g. *Xenorhabdus* sp. y

[15] Proceso 06-IP-89 (http://intranet.comunidadandina.org/Documentos/Procesos/209-IP-2005.doc) y Proceso 36-IP-2004 (http://www.notinet.com.co/serverfiles/servicios/archivos/30jul04/ca36-04.htm)

Photorhabdus sp), se 25 toman individuos juveniles de nematodos entomopatógenos, se inyectan en una larva de *Galleria mellonella* de último instar, a la cual, una vez infectada (entre 2 y 3 días), se le extrae una gota de hemolinfa de la parte inferior de la cabeza. Se siembra en una caja de Petri con medio NBTA que comprende caldo nutritivo, agar, azul de bromotimol, agua y cloruro de trifenil tetrazolium siguiendo la metodología descrita en Stock, P., & Goodrich-Blair, H. (2012). Una vez aisladas las bacterias, se purifican por la metodología de agotamiento o cualquier otra técnica conocida por un técnico en la materia, y se cultivan a las mismas condiciones utilizadas para el aislamiento. Posteriormente se conservan las cepas puras en un congelador entre -80 y -20°C. El cultivo de las bacterias conservadas se realiza en medio de cultivo XG a las condiciones antes mencionadas. Posteriormente, en la preparación del pre-inóculo del microorganismo, consiste este en cultivar las bacterias almacenadas en cajas de Petri con medio de cultivo XG con pH entre 5,5 y 8,0. De allí se toman entre 2 y 5 colonias aisladas y se llevan a caldo nutritivo donde se dejan crecer por un periodo entre 18 y 48 horas a una temperatura entre 20 y 35°C y se agitan entre 90 y 200 rpm.

Una vez obtenido el pre-inóculo continúa la etapa c), donde se inocula un volumen del preinóculo en caldo nutritivo en presencia de entre el 2 y 5% de una resina adsorbente hidrofóbica, en condiciones apropiadas (temperatura entre 20 y 35ºC y agitación entre 100 y 200 rpm) durante 5 a 10 días. Durante la inoculación las bacterias producen los metabolitos secundarios (alrededor del día 5), pero varía dependiendo del microorganismo utilizado. La resina hidrofóbica permite retener los metabolitos secundarios secretados por las bacterias y evita su degradación. Preferiblemente, la resina adsorbente hidrofóbica es una resina hidrofóbica poliaromática es una resina Amberlita XAD 16N®.

Por el contrario, las condiciones de cultivo sugeridas en D1 se refieren a que el microorganismo es incubado por un periodo entre 24 y 96 horas con una velocidad de agitación de 400-600 rpm, aireación de 1 a 5 VVm, temperatura entre 20° C y 40°C., y pH entre 5.5 y 7.5. Un ácido fuerte como ácido sulfúrico y/o una base fuerte como hidróxido de sodio son usados como control y/o para ajustar el pH, y donde se agregan surfactantes y antiespumantes con el fin de controlar la formación de espuma.

c- Sobre el proceso de extracción, en D1 se enseña que a los cultivos obtenidos de *B. subtilis* EA-CB0015 y *B. amyloliquefaciens* EA-CB0959 se les realiza un proceso de extracción con metanol de sus metabolitos activos. Posteriormente, se hace una extracción en fase solida con metanol y las fracciones activas se purifican por HPLC de fase reversa con un detector UV con longitud de onda de 214 nm. Este proceso se realiza con el fin de obtener las fracciones activas y las moléculas responsables de la actividad, el producto se realiza con cultivo bacteriano (contiene células de la bacteria *B. subtilis*).

A su vez, en la presente invención pasado el tiempo de cultivo, se separa y se eluye la resina adsorbente hidrofóbica con un solvente adecuado hasta obtener el extracto bacteriano. Se separa la resina (v.g. por decantación) desechando el medio y luego la resina se transfiere a un recipiente que contiene un volumen de solvente adecuado (entre 5 y 20 mL) para eluir los metabolitos secundarios, lo que puede tomar un tiempo entre 12 y 36 horas. Se entiende por eluir a la aplicación de un solvente o fase móvil que permita arrastrar o separar los componentes que quedaron retenidos en la resina. Entre los solventes se pueden mencionar acetona, metanol, isopropanol y sus mezclas. Una vez eluidos los metabolitos secundarios, se filtra el sobrenadante y se concentran (v.g. en un rotoevaporador o en cualquier equipo que permita evaporar el solvente), hasta un volumen entre 0,5 y 2,0 mL. El extracto bacteriano obtenido se almacena a una temperatura entre 1 y 10°C. La concentración de metabolitos secundarios en los extractos bacterianos obtenidos es superior a 1000 ppm, más preferiblemente, superior a 10000 ppm. La actividad biocida de los extractos obtenidos se puede evaluar sobre diferentes patógenos, por ejemplo, sobre el hongo *L. gongylophorus*. El proceso de extracción se utiliza para la preparación del producto, el cual no contiene biomasa (células bacterianas y/o esporas) de los microorganismos.

d- Sobre el tipo de microorganismos (y como se señalaba en el apartado de novedad del presente memorial) para D1 consiste en un proceso para incrementar la producción de biomasa de microorganismos del género *Bacillus.* Por el contrario en la presente invención el proceso en cuestión no está limitado solo a microorganismos del género *Bacillus*. Los microorganismos que pueden ser utilizados en el proceso de la presente

invención, son 35 aquellos que tengan la capacidad de producir sustancias activas. Bacterias del género *Bacillus* sp, *Serratia* sp, *Pseudomonas* sp, *Burkholderia* sp, *Brevundimonas* sp, *Escherichia* sp, *Delftia* sp, *Acinetobacter* sp, *Photorhabdus* sp y *Xenorhabdus* sp son bacterias asociadas.

e- Respecto a la actividad, en D1 se enseña que la evaluación de *Bacillus subtilis* EA-CB0015 y *Bacillus amyloliquefaciens* EA-CB0959 (sobrenadante libre de células) es realizada frente al hongo fitopatogeno *Mycosphaerella fijiensis* La evaluación se realiza frente a 4 cepas, *M. fijiensis* EASGK09, *M.fijiensis* EASGK10, *M.fijiensis* EASGK11, y *M.fijiensis* EASGK14 mediante la técnica de platos duales con modificaciones. Los porcentajes de inhibición del crecimiento de las colonias de hongos se evaluaron en PDA. Las pruebas de inhibición se realizaron por medición del tubo germinal. En contraste, en la presente invención se determinó la actividad fungicida del extracto bacteriano metanólico obtenido frente al hongo simbionte de la hormiga *Atta cephalotes*. Para esto, se aisló el hongo de los jardines de las hormigas, se tomó una porción de micelio del hongo y se sembró en cajas de Petri con medio PDA acidificado con ácido láctico al 4% a un pH de 4,0. Después de 4 semanas de crecimiento, se tomó una muestra con un sacabocados con un diámetro de 8 mm y se trasladó a una caja de Petri con medio PDA y 400 µL del extracto bacteriano. Se evaluó el crecimiento del micelio por un periodo de 4 semanas. Se realizó un bioensayo con 3 repeticiones y un control, los datos se analizaron estadísticamente con el programa Statsgraphics Centurion® con pruebas de bondad de ajuste, se determinó que de acuerdo con el estadístico log verosimilitud, la distribución de mejor ajuste es la distribución log-logística, con un 95% de confianza. En 5 todos los casos, la eficacia de los tratamientos y las repeticiones se evaluó a través de un análisis de varianza (ANDEVA), concluyendo que los tratamientos difieren en su efecto medio (nivel de significancia $P<0,05$). Además se evalúa el efecto insecticida de los extractos.

f- Finalmente, en lo concerniente al producto, de D1 se motiva un proceso para la producción de biomasa que se integra en una formulación con actividad contra fitopatogenos. Mientras que en la presente invención se desarrolló un Proceso para la

producción de extractos bacterianos (que no contienen biomasa) con actividad antifungica, insecticida y/o repelente de insectos, tal como se reclama en la nueva Reivindicación 1.

7. Finalmente, considero que la presente solicitud cumple con los requisitos de patentabilidad establecidos por la Decisión 486 y por consiguiente solicito respetuosamente que el Despacho proceda con la concesión de la patente.

Atentamente,

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Anexos:

- Capítulo Reivindicatorio Modificado;
- Capítulo Descriptivo Modificado;
- ANEXO 1: A guide to solid phase extraction;
- ANEXO 2: Assessing the effects of adsorptive polymeric resin additions on fungal secondary metabolite chemical diversity.
- ANEXO 3: Overview of the methods for purification of metabolites that are secreted by bacteria
- ANEXO 4: Ficha técnica comercial del producto AMBERLITE XAD16
- ANEXO 5: Solid-Phase Extraction Comparison of (SPE)—a 16 Materials for the Purification of Anthocyanins from Aronia melanocarpa var Nero
- ANEXO 6: Ficha técnico-comercial para las resinas de adsorción "AMBERLITE XAD POLYMERIC RESINS Sigma Prod.
- ANEXO 7: Chemically modified polymeric resins for solid- phase extraction and group separation prior to analysis by liquid or gas chromatography.
- ANEXO 8: Principles and practice of solid-phase extraction

CAPÍTULO REIVINDICATORIO MODIFICADO
(Primer Examen de Fondo)

1. Un proceso para obtener un extracto biocida que comprende las siguientes etapas:

(a) aislar, purificar y cultivar un microorganismo, en donde dicho microorganismo es del género *Bacillus* sp, *Serratia* sp, *Pseudomonas* sp, *Burkholderia* sp, *Brevundimonas* sp, *Escherichia* sp, *Delftia* sp, *Acinetobacter* sp, *Photorhabdus* sp o *Xenorhabdus* sp;

(b) preparar un pre-inóculo del microorganismo;

(c) inocular el pre-inóculo con una resina adsorbente hidrofóbica bajo condiciones ambientales de temperatura, tiempo, pH y agitación en los siguientes rangos:

Condición	**Rango**
Temperatura (°C)	20 a 35
Tiempo (horas)	120 a 240
pH	5,5 a 8,0
Agitación (rpm)	100 a 200

(d) separar y eluir la resina con un solvente orgánico hasta obtener el extracto bacteriano.

2. El proceso según la Reivindicación 1, donde el microorganismo es de la especie *Bacillus mojavensis, Bacillus thuringiensis/cereus, Bacillus amyloliquefaciens ss amyloliquefaciens, Bacillus oleronius, Serratia liquefaciens, Serratia liquefaciens/grimesii, Serratia marcescens ss marcescens, Pseudomonas aeruginosa, Pseudomonas chlororaphis ss aurantiaca, Pseudomona marginalis, Pseudomonas nitroreducens, Pseudomonas tolaasii, Burkholderia dolosa, Brevundimonas diminuta, Escherichia hermannii, Delftia tsuruhatensis, Acinetobacter beijerinckii o Xenorhabdus nematophila.*

3. El proceso según la Reivindicación 1, donde la resina de la etapa (d) es una resina adsorbente de tipo polimérico.

4. El proceso según la Reivindicación 1, donde la elución de la etapa (d) se realiza con acetona, metanol, isopropanol y/o sus mezclas.

5. Un extracto biocida obtenido según el proceso de las Reivindicaciones 1 a 4.

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PROCESO PARA OBTENER UN EXTRACTO BIOCIDA

CAMPO DE LA INVENCIÓN

La invención se enmarca en el campo de los bioprocesos, particularmente en procesos para obtener extractos bacterianos con actividad biocida.

DESCRIPCIÓN DEL ESTADO DE LA TÉCNICA

Se han reportado varios métodos de obtención de extractos activos a partir de microorganismos. El documento US5494809 describe un método para obtener metabolitos secundarios (Iturina A) con actividad antifúngica a partir de bacterias del género *Bacillus* (*Bacillus amyloliquefaciens*), para lo cual inicialmente se cultiva el microorganismo en un medio de cultivo bajo condiciones aeróbicas a 30°C y agitación de 150 rpm. Luego, el medio de cultivo se filtra para separar la biomasa y la fracción filtrada se pasan por una columna hidrofóbica adsorbente ODS C-18.

El documento US20160073642 describe un proceso para incrementar la producción de biomasa de microorganismos del género *Bacillus*, entre ellos *Bacillus subtilis* y *Bacillus amyloliquefaciens*, así como la de sus metabolitos biológicamente activos (lipopéptidos de la familia de las surfactinas, iturinas y fengicinas). Los metabolitos activos se extraen mediante procedimientos de extracción con solventes, precipitación, adsorción o cromatografía.

El documento US9125419 divulga una cepa de *Bacillus* (*Bacillus* sp. F727) aislada y sus metabolitos con actividad biocida. El microorganismo se cultiva empleando un medio de cultivo convencional. Los metabolitos secundarios se extraen del cultivo bacteriano con una resina adsorbente hidrofóbica (Amberlite XAD-7). La resina y las células filtradas se dejan durante 2 horas en acetona, después de lo cual se filtra y se seca al vacío hasta obtener el extracto bacteriano crudo, del cual se pueden obtener fracciones con actividad fungicida.

Se han reportado estudios para utilizar las bacterias *Bacillus mojavensis, Xenorhabdus nematophila, Xenorhabdus* sp. y *Photorhabdus* sp., en programas de control de diferentes hongos y protistas generalmente causales de enfermedades en diferentes cultivos. San-Blas (2012) [1] reporta que *Xenorhabdus* sp. y *Photorhabdus* sp., en cultivos axénicos, tienen una acción antifúngica de hasta el 97% sobre el hongo *Moniliophthora roreri*, agente causal de una de las enfermedades más devastadoras en el cultivo del cacao (*Theobroma cacao*). Por su parte, Fang (2014)[2] determinó la capacidad antimicrobiana de *Xenorhabdus nematophila* contra

[1]San-Blas, E., Carrillo, Z., & Parra, Y. (2012). Effect of *Xenorhabdus* and *Photorhabdus* bacteria and their exudates on *Moniliophthora roreri*. *Archives Of Phytopathology And Plant Protection*, *45*(16), 1[1]950–1967. doi:10.1080/03235408.2012.718688.

[2] Fang, X., Zhang, M., Tang, Q., Wang, Y., & Zhang, X. (2014). Inhibitory effect of *Xenorhabdus nematophila* TB on plant pathogens *Phytophthora capsici* and *Botrytis cinerea* in vitro and in planta. *Scientific Reports*, *4*, 4300. doi:10.1038/srep04300

Botrytis cinerea y *Phytophthora capsici*, dos microorganismos causantes de diversas enfermedades en cultivos de hortalizas alrededor del mundo. Los autores probaron los extractos (metabolitos libres de células bacterianas) contra estos patógenos, encontrando que causan una protección a las plantas hasta de un 70%.

La necesidad permanente de buscar nuevos compuestos para satisfacer la demanda de productos con actividad biocida, ha conllevado a los investigadores a desarrollar un proceso más eficiente de obtención de extractos biocidas a partir de diferentes tipos de microorganismos, de forma tal que se amplíe su espectro de acción y sea más viable su escalado.

BREVE DESCRIPCIÓN DE LAS FIGURAS

FIG. 1 Fotografías correspondientes a diferentes tiempos de observación del extracto UNCSAB03-07-05, el lado izquierdo corresponde al papel filtro con extracto bacteriano, lado derecho corresponde a papel filtro con agua destilada.

FIG. 2 Gráfica del porcentaje de participación de extractos que causan la muerte al 100% de las hormigas a las 24, 48 y más de 48 horas.

BREVE DESCRIPCIÓN DE LA INVENCIÓN

La presente invención se refiere a un proceso para obtener extractos biocidas a partir de microorganismos del género *Bacillus* sp, *Serratia* sp, *Pseudomonas* sp, *Burkholderia* sp, *Brevundimonas* sp, *Escherichia* sp, *Delftia* sp, *Acinetobacter* sp, *Photorhabdus* sp y *Xenorhabdus* sp, que comprende las etapas de aislamiento y cultivo del microorganismo; preparación de un pre-inóculo y ponerlo en contacto con una resina adsorbente hidrofóbica, y por último, eluir con un solvente orgánico hasta obtener el extracto. Los extractos obtenidos exhiben actividad fungicida, insecticida y/o de repelencia.

DESCRIPCIÓN DETALLADA DE LA INVENCIÓN

Los microorganismos que pueden ser utilizados en el proceso de la presente invención, son aquellos que tengan la capacidad de producir sustancias activas. Bacterias del género *Bacillus* sp, *Serratia* sp, *Pseudomonas* sp, *Burkholderia* sp, *Brevundimonas* sp, *Escherichia* sp, *Delftia* sp, *Acinetobacter* sp, *Photorhabdus* sp *y Xenorhabdus* sp son bacterias asociadas a nematodos entomopatógenos y patógenas de insectos que producen metabolitos secundarios con actividad biocida.

El proceso para obtener extractos bacterianos a partir de dichas bacterias comprende las etapas de:

a) aislar, purificar y cultivar el microorganismo;

b) preparar un pre-inóculo del microorganismo;

c) poner en contacto el pre-inóculo con una resina adsorbente hidrofóbica bajo condiciones ambientales adecuadas; y

d) separar y eluir la resina con un solvente orgánico hasta obtener el extracto.

La etapa a) del proceso comprende el aislamiento, purificación y cultivo del microorganismo. Para el aislamiento, se colectan individuos de la hormiga *Atta cephalotes*, se maceran en un tubo de ensayo conteniendo esferas de vidrio y agua peptonada, se agitan a 2500-5000 rpm, se realizan diluciones seriadas entre 10^{-1} y 10^{-8}, se siembran en cajas de Petri con medio de cultivo y se incuban a una temperatura entre 20 y 35°C.

El medio de cultivo debe contener fuentes de carbono y nitrógeno o los nutrientes necesarios para el crecimiento del microorganismo. En una modalidad preferida, el medio de cultivo comprende al menos una fuente de carbono, una fuente de nitrógeno, sales, buffers y nutrientes.

Para el aislamiento de los microorganismos (v.g. *Xenorhabdus* sp. y *Photorhabdus* sp), se toman individuos juveniles de nematodos entomopatógenos, se inyectan en una larva de *Galleria mellonella* de último instar, a la cual, una vez infectada (entre 2 y 3 días), se le extrae una gota de hemolinfa de la parte inferior de la cabeza. Se siembra en una caja de Petri con medio NBTA que comprende caldo nutritivo, agar, azul de bromotimol, agua y cloruro de trifenil tetrazolium siguiendo la metodología descrita en Stock, P., & Goodrich-Blair, H[3]. (2012).

Una vez aisladas las bacterias, se purifican por la metodología de agotamiento o cualquier otra técnica conocida por un técnico en la materia, y se cultivan a las mismas condiciones utilizadas para el aislamiento. Posteriormente se conservan las cepas puras en un congelador entre -80 y -20°C. El cultivo de las bacterias conservadas se realiza en medio de cultivo XG a las condiciones antes mencionadas.

La etapa b), denominada preparación del pre-inóculo del microorganismo, consiste en cultivar las bacterias almacenadas en cajas de Petri con medio de cultivo XG con pH entre 5,5 y 8,0. De allí se toman entre 2 y 5 colonias aisladas y se llevan a caldo nutritivo donde se dejan crecer por un periodo entre 18 y 48 horas a una temperatura entre 20 y 35°C y se agitan entre 90 y 200 rpm. El caldo nutritivo debe contener peptona, extracto de carne, extracto de levadura, cloruro de sodio y/o los nutrientes necesarios para permitir el desarrollo del microorganismo. En una modalidad preferida, el caldo nutritivo tiene extracto de carne 1,0 g, extracto de levadura 2,0 g,

[3]Stock, P., & Goodrich-Blair, H. (2012). Nematode parasites, pathogens and associates of insects and invertebrates of economic importance. In L. A. Lacey (Ed.), *Manual of Techniques in Invertebrate Pathology* (Second edi., pp. 373 – 423). Academic Press.

peptona 5,0 g y cloruro de sodio 5,0 g.

Una vez obtenido el pre-inóculo continúa la etapa c), donde se inocula un volumen del pre-inóculo en caldo nutritivo en presencia de entre el 2 y 5% de una resina adsorbente hidrofóbica, en condiciones apropiadas (temperatura entre 20 y 35ºC y agitación entre 100 y 200 rpm) durante 5 a 10 días. Durante la inoculación las bacterias producen los metabolitos secundarios (alrededor del día 5), pero varía dependiendo del microorganismo utilizado. La resina hidrofóbica permite retener los metabolitos secundarios secretados por las bacterias y evita su degradación. Preferiblemente, la resina adsorbente hidrofóbica es una resina hidrofóbica poliaromática es una resina Amberlita XAD 16N®.

Finalmente se separa y se eluye la resina adsorbente hidrofóbica con un solvente adecuado hasta obtener el extracto bacteriano. Se separa la resina (v.g. por decantación) desechando el medio y luego la resina se transfiere a un recipiente que contiene un volumen de solvente adecuado (entre 5 y 20 mL) para eluir los metabolitos secundarios, lo que puede tomar un tiempo entre 12 y 36 horas. Se entiende por eluir a la aplicación de un solvente o fase móvil que permita arrastrar o separar los componentes que quedaron retenidos en la resina. Entre los solventes se pueden mencionar acetona, metanol, isopropanol y sus mezclas.

Una vez eluidos los metabolitos secundarios, se filtra el sobrenadante y se concentran (v.g. en un rotoevaporador o en cualquier equipo que permita evaporar el solvente), hasta un volumen entre 0,5 y 2,0 mL. El extracto bacteriano obtenido se almacena a una temperatura entre 1 y 10°C. La concentración de metabolitos secundarios en los extractos bacterianos obtenidos es superior a 1000 ppm, más preferiblemente, superior a 10000 ppm. La actividad biocida de los extractos obtenidos se puede evaluar sobre diferentes patógenos, por ejemplo, sobre el hongo *L. gongylophorus*.

Los siguientes Ejemplos ilustran la invención, sin estar el concepto inventivo restringido a los mismos.

EJEMPLOS

Ejemplo 1. Aislamiento, purificación y cultivo de *Bacillus mojavensis* UNCSAB02-24 aislado de una hormiga (*Atta cephalotes*) cortadora de hojas

Se tomaron 6 hormigas cortadoras en un tubo de ensayo estéril con esferas de vidrio de 6 mm de diámetro y se agregó agua peptonada hasta cubrir en su totalidad los insectos. Se agitaron en un agitador vortex a 3000 rpm a velocidad constante por un minuto a temperatura ambiente. Luego se realizaron diluciones seriadas y se sembraron las diluciones 10^{-3} y 10^{-5} en superficie en cajas de Petri con agar nutritivo (medio de cultivo) por un periodo de 24 horas a 30°C.

Las bacterias obtenidas se aislaron y se purificaron por la metodología de agotamiento en las mismas condiciones. Posteriormente, se conservaron las cepas puras en micro-tubos con 70% de caldo nutritivo y 30% de glicerol a 99% en un congelador a -20°C. Se obtuvo un cultivo puro de *Bacillus mojavensis.*

Ejemplo 2. Preparación del pre-inóculo de *Bacillus mojavensis* UNCSAB02-24, inoculación y obtención del extracto

El cultivo puro obtenido según el Ejemplo 1 y almacenado a -20°C, se cultivó en cajas de Petri con medio XG con un pH 7,0. Se hizo un pre-inóculo a partir de este cultivo en el que se tomaron 3 colonias aisladas y se colocaron en 5 mL de caldo nutritivo, se dejaron por 24 horas a 30°C y 150 rpm.

Para la preparación del extracto se inocularon 100 µ L de este cultivo y se transfirieron a un Erlenmeyer con 100 mL de caldo nutritivo y en presencia de 2,5 mL de resina Amberlita XAD 16N en solución acuosa obtenidos a partir de la mezcla de 50g de resina y 50mL de agua estéril, se esterilizaron. Se incubó por 7 días a 30°C y 150 rpm. Posteriormente se descartó el medio y se recuperó la resina, la cual se transfirió a 15 mL de metanol al 99,99% y se dejó eluir por 24 horas. Se filtró el sobrenadante y se concentró en un rotoevaporador hasta obtener aproximadamente 1 mL de extracto bacteriano metanólico. El extracto obtenido se almacenó a 4ºC.

Ejemplo 3. Evaluación del efecto antifúngico del extracto de *Bacillus mojavensis* UNCSAB02-24 contra *Leucoagaricus gongylophorus*

Se determinó la actividad fungicida del extracto bacteriano metanólico obtenido en el Ejemplo 2 frente al hongo simbionte de la hormiga *Atta cephalotes*. Para esto, se aisló el hongo de los jardines de las hormigas, se tomó una porción de micelio del hongo y se sembró en cajas de Petri con medio PDA acidificado con ácido láctico al 4% a un pH de 4,0. Después de 4 semanas de crecimiento, se tomó una muestra con un sacabocados con un diámetro de 8 mm y se trasladó a una caja de Petri con medio PDA y 400 µL del extracto bacteriano. Se evaluó el crecimiento del micelio por un periodo de 4 semanas.

Se realizó un bioensayo con 3 repeticiones y un control, los datos se analizaron estadísticamente con el programa Statsgraphics Centurion® con pruebas de bondad de ajuste, se determinó que de acuerdo con el estadístico log verosimilitud, la distribución de mejor ajuste es la distribución log-logística, con un 95% de confianza. En todos los casos, la eficacia de los tratamientos y las repeticiones se evaluó a través de un análisis de varianza (ANDEVA), concluyendo que los tratamientos difieren en su efecto medio (nivel de significancia $P<0,05$). Los resultados se

muestran en las Tablas 1 y 2.

Tabla 1. Resultados ANDEVA obtenidos para el bioensayo

Grupos	Cuenta	Suma	Porcentaje de inhibición	Varianza
UNCSAB02-01	2	64,39	32,19	5,39
UNCSAB02-02	1	4,88	4,88	0,0
UNCSAB02-04	3	142,70	47,57	410,72
UNCSAB02-05	2	61,90	30,95	310,79
UNCSAB02-06	2	37,77	18,88	396,89
UNCSAB02-14	3	24,50	8,17	133,20
UNCSAB02-16	3	63,64	21,21	433,01
UNCSAB02-18	3	69,84	23,28	333,77
UNCSAB02-19	2	73,31	36,66	149,66
UNCSAB02-21	2	48,98	24,49	475,91
UNCSAB02-21A	3	67,54	22,51	192,59
UNCSAB02-23	3	78,02	26,01	429,32
UNCSAB02-24	3	322,15	107,38	1,43
UNCSAB02-26	3	131,31	43,77	651,57
UNCSAB02-27	2	28,62	14,31	321,99

Tabla 2. Análisis de varianza del bioensayo fungicida

Origen de las variaciones	Suma de cuadrados	Grados de libertad	Promedio de los cuadrados	F	P	Valor crítico para F
Entre grupos	22679,15	14,00	1619,9	5,22	3,05E-04	2,17
Dentro de los grupos	6831,86	22,00	310,5			
Total	29511,01	36,00				

El extracto bacteriano metanólico (92000 ppm) obtenido a partir de *Bacillus mojavensis* UNCSAB02-24 exhibió un porcentaje de inhibición del 100% contra el hongo *Leucoagaricus gongylophorus.*

Ejemplo 4. Aislamiento, purificación y cultivo de *Xenorhabdus nematophila* UNCSAB03-25-01 y *Xenorhabdus* sp. UNCSAB03-23-04 aislados del nematodo *Steinernema* sp

Se tomó una larva de último instar de *Galleria mellonella* y se inoculó con 200 juveniles infectivos del nematodo. Posteriormente, a los 3 días se esterilizó la larva en alcohol al 70% por 5 minutos y se pasó por agua destilada estéril. Luego con la ayuda de una jeringa se rompió el integumento debajo de la cabeza del insecto y se tomó una gota de la hemolinfa, se sembró en caja de Petri con medio NBTA por un periodo de 48 horas a 25°C.

Una vez aisladas las bacterias, se realizó el procedimiento expuesto en el Ejemplo 1 para la purificación y cultivo de *Xenorhabdus nematophila* UNCSAB03-25-01 y *Xenorhabdus nematophila* UNCSAB03-23-04. Se obtuvo un cultivo puro de *Xenorhabdus nematophila* UNCSAB03-25-01 y un cultivo puro de *Xenorhabdus nematophila* UNCSAB03-23-04.

Ejemplo 5. Preparación del pre-inóculo de *Xenorhabdus nematophila* UNCSAB03-25- 01 y *Xenorhabdus* sp. UNCSAB03-23-04. inoculación y obtención del extracto

Se realizó el mismo procedimiento expuesto en el Ejemplo 2 para la preparación del pre- inóculo de *Xenorhabdus nematophila* UNCSAB03-25-01 y *Xenorhabdus* sp. UNCSAB03- 23-04 obtenidos mediante el Ejemplo 4.

Se obtuvo 1 mL de extracto bacteriano metanólico producido a partir de *Xenorhabdus nematophila* UNCSAB03-25-01 y *Xenorhabdus* sp. UNCSAB03-23-04. El extracto obtenido se almacenó a 4ºC.

Ejemplo 6. Aislamiento, purificación y conservación de *Photorhabdus* sp. UNCSAB03- 24-01

Se realizó el procedimiento expuesto en el Ejemplo 4 para el aislamiento, purificación y conservación de *Photorhabdus* sp. UNCSAB03-24-01 aislado del nematodo *Heterorhabditis* sp. Se infectó la larva de *Galleria mellonella* con 200 juveniles infectivo de nematodos, se esperaron entre 24 y 48 horas para realizar una punción cerca a la cabeza de la larva y obtener una gota de hemolinfa, la cual se siembra en el agar NBTA. Una vez aisladas las bacterias, se realizó el procedimiento expuesto en el Ejemplo 1 para la purificación y cultivo de *Photorhabdus* sp. UNCSAB03-24-01. Se obtuvo un cultivo puro de *Photorhabdus* sp. UNCSAB03-24-01.

Ejemplo 7. Preparación del pre-inóculo de *Photorhabdus* sp. UNCSAB03-24-01, inoculación y obtención del extracto

Se realizó el procedimiento expuesto en el Ejemplo 2 para la preparación del pre-inóculo de *Photorhabdus* sp. UNCSAB03-24-01 obtenido mediante el Ejemplo 6. Se obtuvo 1 mL de extracto bacteriano metanólico a partir de *Photorhabdus* sp. UNCSAB03-24-01. El extracto obtenido se almacenó a 4ºC.

Ejemplo 8. Aislamiento, purificación y cultivo de *Bacillus thuringiensis/cereus* UNCSAB03-03-06, *Bacillus amyloliquefaciens ss amyloliquefaciens* UNCSAB03-03-12, *Serratia marcescens ss marcescens* UNCSAB03-04-01, *Bacillus oleronius* UNCSAB03- 04-03, *Serratia marcesens ss marcesens* UNCSAB03-04-05, *Burkholderia dolosa* UNCSAB03-07-04, *Pseudomonas chlororaphis ss aurantiaca* UNCSAB03-07-05, *Brevundimonas diminuta* UNCSAB03-09-04, *Pseudomonas aeruginosa* UNCSAB03-13- 01, *Pseudomonas tolaasii* UNCSAB03-16-01, *Pseudomonas aeruginosa* UNCSAB03-16- 02, *Pseudomonas aeruginosa* UNCSAB03-16-03, *Pseudomona marginalis* UNCSAB03- 18-04, *Serratia liquefaciens* UNCSAB03-19-01, *Pseudomonas aeruginosa* UNCSAB03- 20-01, *Escherichia hermannii* UNCSAB03-21-02, *Serratia liquefasciens/ grimesii* UNCSAB03-21-03, *Pseudomonas nitroreducens* UNCSAB03-21-06, *Serratia marcescens ss marcescens* UNCSAB03-22-01, *Delftia tsuruhatensis* UNCSAB03-22-02, *Acinetobacter beijerinckii* UNCSAB03-22-05, *Serratia marcescens ss marcescens* UNCSAB03-23-01, *Photorhabdus* sp. UNCSAB03-24-01, *Xenorhabdus nematophila* UNCSAB03-25-01

Se tomó una larva de último instar de *Galleria mellonella* y se inoculó con 200 juveniles infectivos del nematodo. Posteriormente, a los 3 días se pasó por agua destilada estéril. Se obtiene un concentrado del cual se tomaron 100µ L de nematodos juveniles, en un tubo de microcentrífuga se maceraron y se realizaron diluciones seriadas y se sembraron las diluciones 10^{-3} y 10^{-5} en superficie en cajas de Petri con agar nutritivo (medio de cultivo) por un periodo de 24 horas a 30°C.

Las bacterias obtenidas se aislaron y se purificaron por la metodología de agotamiento en las mismas condiciones. Posteriormente, se conservaron las cepas puras en micro-tubos con 70% de caldo nutritivo y 30% de glicerol a 99% en un congelador a -20°C.

Ejemplo 9. Preparación del pre-inóculo de *Bacillus thuringiensis/cereus* UNCSAB03- 03-06, *Bacillus amyloliquefaciens ss amyloliquefaciens* UNCSAB03-03-12, *Serratia marcescens ss marcescens* UNCSAB03-04-01, *Bacillus oleronius* UNCSAB03-04-03, *Serratia marcesens ss marcesens* UNCSAB03-04-05, *Burkholderia dolosa* UNCSAB03- 07-04, *Pseudomonas chlororaphis ss aurantiaca* UNCSAB03-07-05, *Brevundimonas diminuta* UNCSAB03-09-04, *Pseudomonas aeruginosa* UNCSAB03-13- 01, *Pseudomonas tolaasii* UNCSAB03-16-01, *Pseudomonas aeruginosa* UNCSAB03-16- 02, *Pseudomonas aeruginosa* UNCSAB03-16-03, *Pseudomona marginalis* UNCSAB03- 18-04, *Serratia liquefaciens* UNCSAB03-19-01, *Pseudomonas aeruginosa* UNCSAB03-20-01, *Escherichia hermannii* UNCSAB03-21-02,

Serratia liquefasciens/ grimesii **UNCSAB03-21-03, *Pseudomonas nitroreducens* UNCSAB03-21-06, *Serratia marcescens ss marcescens* UNCSAB03-22-01, *Delftia tsuruhatensis* UNCSAB03-22-02, *Acinetobacter beijerinckii* UNCSAB03-22-05, *Serratia marcescens ss marcescens* UNCSAB03-23-01, *Photorhabdus* sp. UNCSAB03-24-01, *Xenorhabdus nematophila* UNCSAB03-25-01**

El cultivo puro obtenido en el Ejemplo 8 y almacenado a -20°C, se cultivó en cajas de Petri con medio agar nutritivo con un pH 7,5. Se hizo un pre-inóculo a partir de este cultivo en el que se tomaron 3 colonias aisladas y se colocaron en 5 mL de caldo nutritivo, se dejaron por 24 horas a 27°C y 120 rpm.

Para la preparación del extracto se inocularon 100 µ L de este cultivo y se transfirieron a un Erlenmeyer con 100 mL de caldo nutritivo y en presencia de 2,5 mL de resina Amberlita XAD 16N en solución acuosa obtenidos a partir de la mezcla de 50g de resina y 50mL de agua estéril, se esterilizaron. Se incubó por 7 días a 27°C y 120 rpm. Posteriormente se descartó el medio y se recuperó la resina por decantación, la cual se transfirió a 15 mL de metanol al 99,99% y se dejó eluir por 24 horas, se filtró el sobrenadante y se concentró en un rotoevaporador hasta obtener aproximadamente 2 mL de extracto bacteriano metanólico producido a partir de cada una de las especies. El extracto obtenido se almacenó a 4ºC.

Ejemplo 10. Efecto antifúngico del extracto de *Xenorhabdus nematophila* UNCSAB03- 25-01, *Xenorhabdus nematophila* UNCSAB03-23-04 y *Photorhabdus* sp. UNCSAB03- 24-01 contra *Leucoagaricus gongylophorus*, *Burkholderia dolosa* UNCSAB03-07-04; *Pseudomonas chlororaphis ss aurantiaca* UNCSAB03-07-05, *Pseudomonas aeruginosa* UNCSAB03-16-02, *Pseudomonas aeruginosa* UNCSAB03-16-03, *Pseudomona marginalis* UNCSAB03-18-04.

Se determinó la efectividad de los extractos bacterianos metanólicos obtenidos según el Ejemplo 5, Ejemplo 7 y Ejemplo 9, frente al hongo simbionte de la hormiga *Atta cephalotes* siguiendo el procedimiento expuesto en el Ejemplo 3:

Se realizó un bioensayo con 3 repeticiones y un testigo, donde las mediciones se realizaron cada 7 días por cinco semanas calculando el área de crecimiento con la ayuda de un programa de procesamiento científico de imágenes, particularmente programa ImageJ versión 1.49q (Rasband, n.d.). Los valores del área bajo la curva de crecimiento para las tres bacterias en promedio, la desviación estándar y la comparación entre cada extracto restando con el testigo (Mediante una prueba de Dunett) se exponen en la Tabla 3 y Tabla 4, las diferencias significativas se resaltan con un *:

Tabla 3. Promedios y desviación estándar de cada extracto obtenido

Extracto bacteriano	Promedio	Des. estándar
UNCSAB03-03-11	47,137	0,767
UNCSAB03-03-12	70,453	4,621
UNCSAB03-07-04	35,026	3,903
UNCSAB03-07-05	35,670	3,701
UNCSAB03-10-02	59,485	1,204
UNCSAB03-12-02	60,391	2,970
UNCSAB03-13-03	56,776	2,873
UNCSAB03-14-03	59,640	2,140
UNCSAB03-15-02	52,470	2,290
UNCSAB03-15-07	56,617	8,380
UNCSAB03-16-02	34,827	5,802
UNCSAB03-16-03	33,804	1,298
UNCSAB03-16-04	40,637	3,986
UNCSAB03-16-05	43,619	8,198
UNCSAB03-18-04	32,095	5,821
UNCSAB03-19-03	52,968	3,023
UNCSAB03-23-04	16,680	0,023
UNCSAB03-24-01	23,041	3,225
UNCSAB03-25-01	15,265	0,663
Testigo	46,227	5,119

Tabla 4. Comparaciones de medias, bioensayo fungicida, en la casilla de comparación es cada extracto restado con el testigo (negativo)

Comparación	Promedios
UNCSAB03-03-12 - Testigo	24,225*
UNCSAB03-12-02 - Testigo	14,164*
UNCSAB03-14-03 - Testigo	13,413*
UNCSAB03-10-02 - Testigo	13,258*
UNCSAB03-13-03 - Testigo	10,549*
UNCSAB03-15-07 - Testigo	10,390*
UNCSAB03-19-03 - Testigo	6,741
UNCSAB03-15-02 - Testigo	6,242
UNCSAB03-03-11 - Testigo	0,910
UNCSAB03-16-05 - Testigo	-2,608
UNCSAB03-16-04 - Testigo	-5,590
UNCSAB03-07-05 - Testigo	-10,558*

UNCSAB03-07-04 - Testigo	-11,202*
UNCSAB03-16-02 - Testigo	-11,400*
UNCSAB03-16-03 - Testigo	-12,423*
UNCSAB03-18-04 - Testigo	-14,132*
UNCSAB03-24-01 - Testigo	-23,187*
UNCSAB03-23-04 - Testigo	-29,547*
UNCSAB03-25-01 - Testigo	-30,963*

En el test de Dunnett se puede afirmar con una confiabilidad del 95% que la diferencia mínima significativa para que un tratamiento sea diferente al testigo debe tener un valor mínimo de 10,353 cm^2, lo que confirma que los extractos ensayados causaron una inhibición del crecimiento del hongo estadísticamente significativa.

Los porcentajes de inhibición contra el hongo *Leucoagaricus gongylophorus* exhibidos por los extractos bacterianos metanólicos obtenidos en este Ejemplo están entre el 25% y el 80%, se resalta el porcentaje de inhibición: 79% *Xenorhabdus nematophila* UNCSAB03-25-01, 78% *Xenorhabdus* sp UNCSAB03-23-04, 58% *Photorhabdus* sp. UNCSAB03-24- 01 y 41% *Pseudomona marginalis* UNCSAB03-18-04. Las partes por millón (ppm) contenidas en los extractos bacterianos con un total de 50500 ppm en UNCSAB03-25-01, 32000 ppm en UNCSAB03-24-01.

Ejemplo 11. Efecto de repelencia del extracto de *Bacillus amyloliquefaciens ss amyloliquefaciens* UNCSAB03-03-12, *Pseudomonas chlororaphis ss aurantiaca* UNCSAB03-07-05, *Pseudomonas aeruginosa* UNCSAB03-20-01, *Pseudomonas nitroreducens* UNCSAB03-21-06, *Serratia marcescens ss marcescens* UNCSAB03-22- 01, *Delftia tsuruhatensis* UNCSAB03-22-02, *Serratia marcescens ss marcescens* UNCSAB03-23-01, *Xenorhabdus nematophila* UNCSAB03-23-04, *Xenorhabdus nematophila* UNCSAB03-25-01.

Para el bioensayo por repelencia se usaron dos papeles filtro separados por 0,5 cm entre ellos, cada uno a un lado del fondo de una caja de Petri de Ø 90 mm. En uno de los papeles filtro se adicionó el extracto bacteriano y en el otro el agua destilada.

Las mediciones se realizaron cada 5 minutos por 60 minutos, contando las hormigas a cada lado en el periodo de tiempo exacto, se registró con la ayuda de una cámara fotográfica. Los extractos inicialmente se encontraban suspendidos en metanol, sin embargo, para los experimentos de Bioensayo insecticida por contacto y Bioensayo por repelencia se tomó la cantidad del extracto suspendido en metanol y se procedió a evaporarlo con el fin de suspender el extracto bacteriano en agua destilada.

Los datos del bioensayo de repelencia se dividen por observaciones cada cinco minutos (FIG. 1) durante una hora, para lo cual cada una de las observaciones puede analizarse como una prueba de preferencia, usando la prueba del signo para la comparación de muestras pareadas y su interpretación. Este bioensayo dio como resultado ocho extractos con efecto repelente significativo hacia las obreras de *Atta cephalotes*.

Si se define *P* como la probabilidad de que una hormiga se ubique en el sector impregnado con el extracto, *x* será el número de hormigas encontradas en el extracto, en una lectura determinada, pudiendo tomar 11 posibles valores: 0, 1, 2, …, 9, 10. Considérese el siguiente juego de hipótesis:

$$H_0 : p \geq 0.5$$
$$H_a : p < 0.5$$

La hipótesis nula plantea que el extracto no tiene efecto repelente (pudiendo, de hecho, ser atrayente). La hipótesis alternativa plantea que el extracto tiene efecto repelente. Solamente, mediante el rechazo de la hipótesis nula podría probarse el efecto repelente del extracto.

Usando un script en R (R Core Team, 2014), puede generarse la siguiente tabla (Tabla 5), con los valores *P* para cada uno de los posibles resultados: este valor *P* es una distribución binomial que se basa en la prueba estadística del signo.

Tabla 5. Valor *P* para los diferentes resultados en el bioensayo de repelencia

Hormigas en el extracto	**Valor *P***	**Límite superior**
0	0,0009766	0,2129
1	0,0107422	0,3477
2	0,0546875	0,4592
3	0,171875	0,5583
4	0,3769531	0,6484
5	0,623047	0,7307
6	0,828125	0,8058
7	0,9453125	0,8731
8	0,9892578	0,9314
9	0,9990234	0,9774
10	1	1

Luego, únicamente puede afirmarse que el extracto tiene un efecto repelente significativo cuando se observa una hormiga o ninguna (valores *P* menores o iguales que 0,05). En tales casos,

puede afirmarse con una confianza del 95% que la probabilidad de encontrar hormigas en el extracto es inferior a 0,5 (efecto repelente). La tercera columna, calculada con base en la función *inf.p* en el software R, muestra el límite superior del estimador por intervalo de dicha probabilidad, obtenidos mediante el método exacto de Clopper-Pearson, de manera que haya coincidencia con la prueba de hipótesis. Este límite superior se puede interpretar como la máxima probabilidad de encontrar una hormiga en futuras ocasiones en el extracto probado.

Así se tiene, por ejemplo, una confianza del 95% de que en los casos en que se observó una hormiga en la porción con extracto, la máxima probabilidad de preferencia sería de 0,3942, lo que implica una inhibición 0,6058. Cuando se observan dos hormigas en el extracto no puede afirmarse con una probabilidad de error inferior a 0,05 que el extracto tenga efecto repelente. En este caso, la verdadera probabilidad de preferencia podría ser tan alta como 0.5069, lo cual implica que no habría ningún tipo de repelencia.

En la Tabla 6 se tabulan los datos del bioensayo repelencia. Los datos corresponden a las hormigas observadas en el lado del extracto en cada tiempo. En verde se marcan los que se encuentran por debajo del 0,05 en el valor *P* por lo tanto son observaciones estadísticamente significativas. La columna de la derecha corresponde a un puntaje dado para interpretar de forma más fácil dicha Tabla 6.

Tabla 6. Datos bioensayo repelencia, los datos corresponden a las hormigas observadas en el lado del extracto en cada tiempo. En verde se marcan los que se encuentran por debajo del 0,05 en el valor P por lo tanto son observaciones estadísticamente significativas. La columna de la derecha corresponde a un puntaje dado para interpretar de forma más fácil la tabla.

DATOS BIOENSAYO REPELENCIA													
Extracto - Timepo	**05**	**10**	**15**	**20**	**25**	**30**	**35**	**40**	**45**	**50**	**55**	**60**	**Sumatoria**
UNCSAB03-22-01	0	0	0	1	1	0	0	0	0	0	0	0	2
UNCSAB03-23-01	0	0	0	0	0	0	0	1	1	0	0	0	2
UNCSAB03-23-04	1	1	1	0	0	0	0	0	0	0	0	0	3
UNCSAB03-20-01	0	0	1	2	0	1	2	1	0	1	1	1	10
UNCSAB03-21-06	1	2	2	2	1	0	1	1	0	0	0	0	10
UNCSAB03-22-02	5	2	0	0	0	1	1	0	1	0	0	0	10
UNCSAB03-25-01	0	2	1	2	2	1	1	0	0	1	0	0	10
UNCSAB03-07-05	1	2	2	1	1	0	1	1	0	0	1	1	11
UNCSAB03-03-12	1	2	1	1	1	2	0	1	3	0	1	0	13
UNCSAB03-04-01	3	5	4	0	1	1	1	1	0	0	0	0	16
UNCSAB03-09-03	1	1	0	1	1	0	4	1	4	1	1	1	16
UNCSAB03-21-02	4	2	0	5	2	2	1	0	0	0	0	0	16

UNCSAB03-03-06	4	2	3	2	2	1	2	0	1	1	0	1	19
UNCSAB03-22-05	2	3	3	2	2	2	2	1	1	1	1	1	21
UNCSAB03-09-04	1	3	2	4	0	3	3	2	2	2	3	1	26
UNCSAB03-21-03	2	1	2	5	2	3	0	4	2	4	1	0	26
UNCSAB03-16-01	3	2	1	2	2	3	2	3	3	2	2	2	27
UNCSAB03-24-01	4	1	3	1	2	3	2	4	3	1	1	2	27
UNCSAB03-13-01	0	3	5	1	1	0	4	2	2	3	4	3	28
UNCSAB03-04-05	5	1	4	1	2	2	6	2	2	0	3	1	29
UNCSAB03-23-02	2	5	4	4	3	4	3	4	2	0	3	5	39
Testigo	4	4	6	9	5	4	0	7	2	5	4	4	54
UNCSAB03-19-01	4	4	5	5	5	5	5	4	6	5	4	4	56
UNCSAB03-12-04	0	8	7	8	6	3	4	3	7	7	4	4	61

Uno de los extractos con mayor actividad en el bioensayo repelencia fue UNCSAB03-23- 01, al cual se determinó que contiene 32000 ppm.

Ejemplo 12. Efecto insecticida por ingestión del extracto de Bacillus thuringiensis/cereus UNCSAB03-03-06, Serratia marcescens ss marcescens UNCSAB03-04-01, Serratia marcescens ss marcescens UNCSAB03-04-05, Serratia marcescens ss marcescens UNCSAB03-23-01, Pseudomonas aeruginosa UNCSAB03-13-01, Pseudomonas tolaasii UNCSAB03-16-01, Serratia liquefaciens UNCSAB03-19-01, Escherichia hermannii UNCSAB03-21-02, Serratia liquefaciens/grimesii UNCSAB03-21-03, Pseudomonas nitroreducens UNCSAB03-21-06, Serratia marcescens ss marcescens UNCSAB03-22-01, Acinetobacter beijerinckii UNCSAB03-22-05, Photorhabdus sp UNCSAB03-24-01.

Para este bioensayo se usó una porción de dieta de 8 mm, la cual, contiene el extracto bacteriano de la siguiente forma: en una caja de Petri de Ø 50 mm se adicionan 10 mL de dieta (descrita en el bioensayo anterior) y se mezclan con 400 µ L de extracto bacteriano, Estos extractos fueron suspendidos en metanol por lo que se dejó la caja de Petri abierta en la cámara de flujo laminar por 10 minutos para que se evaporara el metanol.

La dieta se suministró todos los días y consistió en una porción de 8 mm, lo mismo para el testigo. Se colocaron un total de 5 hormigas obreras por caja de Petri, las mediciones se realizaron cada 24 horas por 5 días contando el número de hormigas muertas por caja de Petri, este bioensayo se realizó por triplicado.

Para el análisis de los datos se utilizó el programa Excel y se compararon los resultados utilizando una técnica adecuada para estos casos, la técnica se denomina área bajo la curva de la mortalidad acumulada, para cada una de las unidades experimentales. Este análisis también se realiza en el bioensayo del Ejemplo 10 y por medio del programa estadístico SAS (SAS

Institute Inc., 2002) se recopiló la información del área bajo la curva, con la cual se realizó un análisis de varianza y se generó la diferencia mínima significativa que debe cumplir cada tratamiento para diferenciarse estadísticamente del testigo, esto por medio de una prueba de DUNNETT.

Para este bioensayo los extractos bacterianos, UNCSAB03-22-05 y UNCSAB03-04-01 lograron matar el 100 % de las hormigas en menos de 48 horas. A las 72 horas después del inicio del bioensayo, se observó que 11 extractos bacterianos (52,3 %) lograron matar un promedio mayor a 4,67 hormigas. A este grupo se pertenecen las bacterias *Photorhabdus* sp. (UNCSAB03-24-01), *Serratia marcescens* sub. *marcescens* UNCSAB03-04-05 y *Pseudomonas tolaasii* UNCSAB03-16-01.

La Tabla 7 muestra los resultados promedio del bioensayo ingestión. Se presenta el promedio de las tres repeticiones, los números representan los promedios de las hormigas muertas acumuladas.

Tabla 7. Resultados promedio del bioensayo ingestión, se presenta el promedio de las tres repeticiones, los números representan los promedios de las hormigas muertas acumuladas.

Bacteria	Dia 1 (24 H)	Dia 2 (48 H)	Dia 3 (72 H)	Dia 4 (96 H)	Dia 5 (120 H)
UNCSAB03-22-05	3,3	5,0	5,0	5,0	5,0
UNCSAB03-04-01	1,0	5,0	5,0	5,0	5,0
UNCSAB03-03-06	0,3	4,7	5,0	5,0	5,0
UNCSAB03-21-03	0,7	3,3	5,0	5,0	5,0
UNCSAB03-16-01	0,0	1,7	5,0	5,0	5,0
UNCSAB03-22-01	1,7	4,7	4,7	5,0	5,0
UNCSAB03-23-01	1,0	4,7	4,7	4,7	4,7
UNCSAB03-12-04	0,3	3,7	4,7	4,7	5,0
UNCSAB03-04-05	0,7	3,3	4,7	5,0	5,0
UNCSAB03-21-06	1,3	3,0	4,7	4,7	4,7
UNCSAB03-24-01	0,0	2,7	4,7	4,7	4,7
UNCSAB03-19-01	1,3	3,7	4,0	4,0	4,0
UNCSAB03-13-01	0,3	3,7	3,7	4,0	4,0
UNCSAB03-21-02	1,7	2,7	3,7	3,7	4,0
UNCSAB03-22-02	1,0	2,0	2,3	2,3	2,3
UNCSAB03-03-12	0,3	1,7	2,3	3,0	3,7
UNCSAB03-09-04	0,3	1,3	2,0	2,0	2,7
UNCSAB03-23-02	0,3	1,7	1,7	2,7	3,3
UNCSAB03-07-05	0,3	1,0	1,7	4,0	4,3
UNCSAB03-25-01	0,0	1,0	1,0	3,0	4,3

UNCSAB03-23-04	0,3	0,3	0,7	4,3	4,7
Control	0,0	0,0	0,3	1,0	1,0

En el análisis de varianza Tabla 8, se puede observar que el valor *P* es menor a 0,05 por lo tanto se puede concluir que de los 21 tratamientos al menos 2 son diferentes. En la Tabla 9 se tabulan los promedios de las tres repeticiones con sus respectivas desviaciones estándar. Es importante recalcar que los valores de las tablas se refieren al área bajo la curva de cada tratamiento. En la Tabla 10 se muestran los resultados del test de Dunnet.

Tabla 8. Análisis de varianza bioensayo insecticida por ingestión, tabla resumen

Fuente	DF	Suma de cuadrados	Cuadrado medio	Valor F	Pr > F
Modelo	21	1367,32	65,11	5,86	<,0001
Error	44	489	11,11		
Total correjido	65	1856,32			

Tabla 9. Promedios y desviación estándar bioensayo ingestión

Bioensayo ingestión			
Extracto	**N**	**Promedio**	**Des. Estandar**
Control	3	1,8333	1,6073
UNCSAB03-03-06	3	17,3333	0,2887
UNCSAB03-03-12	3	9,0000	4,5826
UNCSAB03-04-01	3	18,0000	0,8660
UNCSAB03-04-05	3	15,8333	0,7638
UNCSAB03-07-05	3	9,0000	2,1794
UNCSAB03-09-04	3	6,8333	3,8837
UNCSAB03-12-04	3	15,6667	1,6073
UNCSAB03-13-01	3	13,5000	4,2720
UNCSAB03-16-01	3	14,1667	1,5275
UNCSAB03-19-01	3	14,3333	4,7522
UNCSAB03-21-02	3	12,8333	3,7859
UNCSAB03-21-03	3	16,1667	1,6073
UNCSAB03-21-06	3	15,3333	3,4034
UNCSAB03-22-01	3	17,6667	1,5275
UNCSAB03-22-02	3	8,3333	8,7797
UNCSAB03-22-05	3	19,1667	0,7638
UNCSAB03-23-01	3	16,8333	2,0207
UNCSAB03-23-02	3	7,8333	2,7538
UNCSAB03-23-04	3	7,8333	1,8930
UNCSAB03-24-01	3	14,3333	2,9297

UNCSAB03-25-01	3	7,1667	4,6458

Tabla 10. Tabla de resultados test de Dunnett bioensayo ingestión

Test de Dunnett	
Alpha	0,05
Grados de libertad del error	44
Cuadrado medio del error	11,11364
Valor critico de Dunnett	3,05508
Diferencia minima significativa	8,3158

En la Tabla 11, se muestra la comparación de tratamientos con respecto al testigo del bioensayo insecticida por ingestión. La casilla de comparación corresponde a cada extracto restado con el testigo (negativo). Los extractos que están marcados con asterisco son aquellos que tienen una diferencia estadísticamente significativa con respecto al testigo. Esto se deduce a partir de comparar la diferencia mínima significativa, Tabla 10, con respecto a la columna promedios de la Tabla 11.

Tabla 11. Comparación de tratamientos con respecto al testigo del bioensayo insecticida por ingestión, en la casilla de comparación es cada extracto restado con el testigo (negativo)

Comparación de cada extracto con el testigo	
Comparacion	**Promedio**
UNCSAB03-22-05 - Testigo	17,333***
UNCSAB03-04-01 - Testigo	16,167***
UNCSAB03-22-01 - Testigo	15,833***
UNCSAB03-03-06 - Testigo	15,500***
UNCSAB03-23-01 - Testigo	15,000***
UNCSAB03-21-03 - Testigo	14,333***
UNCSAB03-04-05 - Testigo	14,000***
UNCSAB03-12-04 - Testigo	13,833***
UNCSAB03-21-06 - Testigo	13,500***
UNCSAB03-19-01 - Testigo	12,500***
UNCSAB03-24-01 - Testigo	12,500***
UNCSAB03-16-01 - Testigo	12,333***
UNCSAB03-13-01 - Testigo	11,667***
UNCSAB03-21-02 - Testigo	11,000***
UNCSAB03-03-12 - Testigo	7,167
UNCSAB03-07-05 - Testigo	7,167
UNCSAB03-22-02 - Testigo	6,500
UNCSAB03-23-02 - Testigo	6,000

UNCSAB03-23-04 - Testigo	6,000
UNCSAB03-25-01 - Testigo	5,333
UNCSAB03-09-04 - Testigo	5,000

En la Tabla 12 se puede observar que el valor *P* es menor a 0,05, por lo tanto se puede concluir que de los 21 tratamientos, al menos 2 son diferentes. En la Tabla 13 se muestran los resultados del test de Dunnet.

Tabla 12. Análisis de varianza bioensayo insecticida por ingestión 24 horas, tabla resumen

Fuente	DF	Suma de cuadrados	Cuadrado medio	Valor F	Pr > F
Modelo	21	5,77140	0,2748	2,35	0,0085
Error	44	5,14991	0,1170		
Total correjido	65	10,92131			

Tabla 13. Tabla test de Dunnett bioensayo insecticida por ingestión, 24 horas

Test de Dunnett	
Alpha	0,05
Grados de libertad del error	44
Cuadrado medio del error	0,117043
Valor critico de Dunnett	3,05508
Diferencia minima significativa	0,8534

La Tabla 14 muestra la comparación de tratamientos con respecto al testigo bioensayo insecticida por ingestión, 24 horas. En la casilla de comparación es cada extracto restado con el testigo (negativo).

En la Tabla 14 se puede apreciar que solo el primer extracto UNCSAB03-22-05 está marcado con asterisco, lo cual significa que tiene una diferencia estadísticamente significativa con respecto al testigo. Esto se traduce en que solo este extracto se diferencia del testigo a las primeras 24 horas del bioensayo ya que causó la muerte de más de tres hormigas para este tiempo.

Tabla 14. Comparación de tratamientos con respecto al testigo bioensayo insecticida por ingestión, 24 horas, en la casilla de comparación es cada extracto restado con el testigo (negativo)

Comparación de cada extracto con el testigo	
Comparacion	**Promedio**
UNCSAB03-22-05 - Testigo	12,253***
UNCSAB03-22-01 - Testigo	0,733
UNCSAB03-21-02 - Testigo	0,679
UNCSAB03-21-06 - Testigo	0,636
UNCSAB03-19-01 - Testigo	0,561
UNCSAB03-23-01 - Testigo	0,518
UNCSAB03-22-02 - Testigo	0,464
UNCSAB03-04-01 - Testigo	0,388
UNCSAB03-04-05 - Testigo	0,345
UNCSAB03-21-03 - Testigo	0,345
UNCSAB03-03-12 - Testigo	0,173
UNCSAB03-12-04 - Testigo	0,173
UNCSAB03-13-01 - Testigo	0,173
UNCSAB03-03-06 - Testigo	0,173
UNCSAB03-09-04 - Testigo	0,173
UNCSAB03-07-05 - Testigo	0,173
UNCSAB03-23-02 - Testigo	0,173
UNCSAB03-23-04 - Testigo	0,173
UNCSAB03-16-01 - Testigo	0,000
UNCSAB03-24-01 - Testigo	0,000
UNCSAB03-25-01 - Testigo	0,000

En la Tabla 15 se puede observar que el valor *P* es menor a 0,05 por lo tanto se puede concluir que de los 21 tratamientos al menos dos son diferentes. En la Tabla 16 se muestran los resultados del test de Dunnet.

Tabla 15. Análisis de varianza bioensayo insecticida por ingestión 48 horas, tabla resumen

Fuente	DF	Suma de cuadrados	Cuadrado medio	Valor F	Pr > F
Modelo	21	14,9771	0,7132	4,77	0,0001
Error	44	6,5746	0,1494		
Total correjido	65	21,5517			

Tabla 16. Tabla test de Dunnett bioensayo insecticida por ingestión, 48 horas

Test de Dunnett	
Alpha	0,05
Grados de libertad del error	44
Cuadrado medio del error	0,149423
Valor critico de Dunnett	3,05508
Diferencia minima significativa	0,9642

La Tabla 17 muestra la comparación de tratamientos con respecto al testigo bioensayo insecticida por ingestión, 48 horas. En la casilla de comparación es cada extracto restado con el testigo (negativo). De acuerdo con la Tabla 17, los primeros 13 extractos están marcados con asterisco, lo cual significa que tienen una diferencia estadísticamente significativa con respecto al testigo. A partir del UNCSAB03-24-01, que tiene un promedio de 1,03, el cual es superior a la diferencia mínima significativa de la prueba de Dunnett 0,9642 (Tabla 15), podemos afirmar que si se repite el bioensayo podemos obtener los mismos resultados con un 95% de confiabilidad.

Los dos extractos con mayor actividad en el bioensayo por ingestión fueron UNCSAB03- 22-05 y UNCSAB03-04-01, de los cuales se determinó las partes por millón (ppm) contenidas en el extracto total. UNCSAB03-22-05 con 34000 ppm y UNCSAB03-04-01 con 31000 ppm.

Tabla 17. Comparación de tratamientos con respecto al testigo bioensayo insecticida por ingestión, 48 horas, en la casilla de comparación es cada extracto restado con el testigo (negativo)

Comparación de cada extracto con el testigo	
Comparacion	**Promedio**
UNCSAB03-22-05 - Testigo	1,64***
UNCSAB03-04-01 - Testigo	1,63***
UNCSAB03-22-01 - Testigo	1,56***
UNCSAB03-03-06 - Testigo	1,56***
UNCSAB03-23-01 - Testigo	1,56***
UNCSAB03-13-01 - Testigo	1,30***
UNCSAB03-19-01 - Testigo	1,30***
UNCSAB03-12-04 - Testigo	1,30***
UNCSAB03-04-05 - Testigo	1,24***
UNCSAB03-21-03 - Testigo	1,22***
UNCSAB03-21-06 - Testigo	1,11***
UNCSAB03-21-02 - Testigo	1,07***
UNCSAB03-24-01 - Testigo	1,03***

UNCSAB03-22-02 - Testigo	0,76
UNCSAB03-03-12 - Testigo	0,76
UNCSAB03-23-02 - Testigo	0,73
UNCSAB03-16-01 - Testigo	0,68
UNCSAB03-09-04 - Testigo	0,58
UNCSAB03-07-05 - Testigo	0,46
UNCSAB03-25-01 - Testigo	0,39
UNCSAB03-23-04 - Testigo	0,17

Ejemplo 13. Efecto insecticida por contacto del extracto de *Bacillus thuringiensis/cereus* UNCSAB03-03-06; *Bacillus amyloliquefaciens ss amyloliquefaciens* UNCSAB03-03-12, *Serratia marcescens ss marcescens* UNCSAB03-04-01, Serratia marcesens ss marcesens UNCSAB03-04-05, *Pseudomonas chlororaphis ss aurantiaca* UNCSAB03-07-05, *Brevundimonas diminuta* UNCSAB03-09-04, *Pseudomonas aeruginosa* UNCSAB03-13-01, *Serratia liquefaciens* UNCSAB03-19-01, *Escherichia hermannii* UNCSAB03-21-02, *Serratia liquefaciens/ grimesii* UNCSAB03-21-03, *Pseudomonas nitroreducens* UNCSAB03-21-06, *Serratia marcescens ss marcescens* UNCSAB03-22-01, *Delftia tsuruhatensis* UNCSAB03-22-02, *Acinetobacter beijerinckii* UNCSAB03-22-05, Serratia marcescens ss marcescens UNCSAB03-23-01, *Xenorhabdus nematophila* UNCSAB03- 25-01.

Para este bioensayo se tomó como base la metodología propuesta por Boulogne y colaboradores (2012), para la cual las hormigas entran en contacto con el extracto por medio del papel filtro al fondo de la caja de Petri. Se empleó una caja de Petri de Ø 50 mm en la cual en el fondo de esta se encontraba un papel filtro, al cual se aplicó 500 µL del extracto o de agua destilada (testigo). Se usaron 5 hormigas obreras mayores.

Para asegurar que la muerte no se debió a la falta de alimento, se suministró una dieta artificial[4] (Bueno, Morini, Pagnocca, Hebling, & Silva, 1997) dentro de la caja de Petri. Las mediciones se realizaron cada 24 horas por 5 días contando el número de hormigas muertas por caja de Petri. En este bioensayo se obtuvo como resultado que 5 extractos mataron el 100 % de las hormigas en 24 horas, otros 13 extractos lograron causar la muerte del 100% de las hormigas en menos de 48 horas. La Tabla 18 muestra el resumen de resultados del bioensayo insecticida por contacto. Se muestran los extractos que lograron matar las hormigas en menos de 48 horas. Los números representan las hormigas muertas acumuladas.

Tabla 18. Resumen de resultados del bioensayo insecticida por contacto, se muestran los extractos que lograron matar las hormigas en menos de 48 horas, los números representan las hormigas muertas acumuladas.

BACTERIA	Hormigas muertas acumuladas	
	Día 1 (24 H)	Día 2 (48 H)
UNCSAB03-03-06	5	5
UNCSAB03-04-05	5	5
UNCSAB03-21-02	5	5
UNCSAB03-22-02	5	5
UNCSAB03-22-05	5	5
UNCSAB03-03-12	4	5
UNCSAB03-04-01	4	5
UNCSAB03-23-01	4	5
UNCSAB03-21-06	3	5
UNCSAB03-25-01	3	5
UNCSAB03-07-05	1	5
UNCSAB03-12-04	1	5
UNCSAB03-22-01	1	5
UNCSAB03-23-02	1	5
UNCSAB03-09-04	0	5
UNCSAB03-13-01	0	5
UNCSAB03-19-01	0	5
UNCSAB03-21-03	0	5

En la Tabla 19 están reportados los mejores extractos que logran matar las hormigas en menos de 24 horas a partir de bacterias aisladas de nematodos de la familia *Rhabditidae*.

Tabla 19. Extractos bacterianos que matan las hormigas en menos de 24 horas por contacto

Extractos que matan las hormigas en al menos 24 horas		
Bacteria	**Sitios de colecta**	**Familia del nematodo**
UNCSAB03-03-06	El Retiro (Ant)	Rhabditidae
UNCSAB03-04-05	El Retiro (Ant)	Rhabditidae
UNCSAB03-21-02	Chinchiná (Caldas)	Rhabditidae
UNCSAB03-22-02	Manizales (Caldas)	Rhabditidae
UNCSAB03-22-05	Manizales (Caldas)	Rhabditidae

En el bioensayo de contacto se puede observar que alrededor del 20 % de los extractos tiene una acción insecticida considerable, estos resultados se pueden ver en la Tabla 20, en la cual se muestra el número de extractos que matan las hormigas en tres rangos de tiempo diferentes. Además de esta tabla se puede apreciar en la FIG. 2 de forma más didáctica. La Tabla 20 muestra el porcentaje de participación del número de extractos por tiempo de exposición, bioensayo

contacto.

Tabla 20. Porcentaje de participación del número de extractos por tiempo de exposición, bioensayo contacto

Tiempo después del contacto	Extractos bacterianos	Porcentaje
24 horas	5	5,4%
48 horas	13	14,0%
> 48 horas	75	80,6%

Se determinó la concentración en partes por millón (ppm) de dos de los cinco mejores extractos en este bioensayo, los resultados fueron: UNCSAB-03-06 con 31500 ppm y UNCSAB-03-04-05 39500 ppm.

Ejemplo 14. Características morfológicas de las colonias formadas por las cepas bacterianas.

En las Tablas 21 a 49 se indican las características morfológicas de las cepas de microorganismos de los Ejemplos 3 y 8, así como de las cepas de otros microorganismos cultivados bajo el mismo procedimiento indicado, para obtener extractos con actividad biocida.

Tabla 21. Características Morfológicas *Bacillus mojavensis* (UNCSAB02-24)

Medio	*Forma*	*Elevación*	*Borde*	*Brillo*	*Textura*	*Color*
Agar nutritivo	Redonda	Elevada	Irregular	Brillante	Cremosa	Blanco

Tabla 22. Características Morfológicas *Xenorhabdus nematophila* (UNCSAB03-25-01)

Medio	*Forma*	*Elevación*	*Borde*	*Brillo*	*Textura*	*Color*
NBTA	Redonda	Elevada	Regular	Brillante	Cremosa	Azul

Tabla 23. Características Morfológicas *Xenorhabdus* sp. *(*UNCSAB03-23-04)

Medio	*Forma*	*Elevación*	*Borde*	*Brillo*	*Textura*	*Color*
NBTA	Redonda	Elevada	Regular	Brillante	Cremosa	Azul

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Tabla 24. Características Morfológicas *Photorhabdus* sp. (UNCSAB03-24-01)

Medio	*Forma*	*Elevación*	*Borde*	*Brillo*	*Textura*	*Color*
NBTA	Redonda	Elevada	Regular	Brillante	Cremosa	Café

Tabla 25. Características Morfológicas *Acinetobacter beijerinckii* (UNCSAB03-22-05)

Medio	*Forma*	*Elevación*	*Borde*	*Brillo*	*Textura*	*Color*
Agar nutritivo	Redonda	Elevada	Regular	Brillante	Acuosa	Amarillo

Tabla 26. Características Morfológicas *Bacillus thuringiensis/cereus* (UNCSAB03-03-6)

Medio	*Forma*	*Elevación*	*Borde*	*Brillo*	*Textura*	*Color*
Agar nutritivo	Irregular	Elevada	Irregular	Opaca	Granulosa	Naranja

Tabla 27. Características Morfológicas *Bacillus amyloliquefaciens ss amyloliquefaciens* (UNCSAB03-03-12)

Medio	*Forma*	*Elevación*	*Borde*	*Brillo*	*Textura*	*Color*
Agar nutritivo	Irregular	Elevada	Irregular	Opaca	Granulosa	Naranja

Tabla 28. Características Morfológicas *Bacillus oleronius* (UNCSAB03-04-03)

Medio	*Forma*	*Elevación*	*Borde*	*Brillo*	*Textura*	*Color*
Agar nutritivo	irregular	Plana	irregular	opaca	granulosa	Blanco

Tabla 29. Características Morfológicas *Brevundimonas diminuta* (UNCSAB03-09-04)

Medio	*Forma*	*Elevación*	*Borde*	*Brillo*	*Textura*	*Color*
Agar nutritivo	Irregular	Elevada	Irregular	Brillante	Cremosa	Blanco

Tabla 30. Características Morfológicas *Burkholderia dolosa* (UNCSAB03-07-04)

Medio	*Forma*	*Elevación*	*Borde*	*Brillo*	*Textura*	*Color*
Agar nutritivo	irregular	Elevada	irregular	Brillante	cremosa	Blanco Amarillo

Tabla 31. Características Morfológicas *Delftia tsuruhatensis* (UNCSAB03-22-02)

Medio	*Forma*	*Elevación*	*Borde*	*Brillo*	*Textura*	*Color*
Agar nutritivo	Irregular	Plana	Irregular	Opaca	Cremosa	Blanco

Tabla 32. Características Morfológicas *Escherichia hermannii* (UNCSAB03-21-02)

Medio	*Forma*	*Elevación*	*Borde*	*Brillo*	*Textura*	*Color*
Agar nutritivo	Redonda	Elevada	Regular	Brillante	Cremosa	Blanco Amarillo

Tabla 33. Características Morfológicas *Photorhabdus* sp. (UNCSAB03-24-01)

Medio	*Forma*	*Elevación*	*Borde*	*Brillo*	*Textura*	*Color*
NBTA	redonda	Elevada	regular	Brillante	cremosa	Café

Tabla 34. Características Morfológicas *Pseudomonas aeruginosa* (UNCSAB03-13-01)

Medio	*Forma*	*Elevación*	*Borde*	*Brillo*	*Textura*	*Color*
Agar nutritivo	Redonda	Elevada	Regular	Brillante	Cremosa	Blanco

Tabla 35. Características Morfológicas *Pseudomonas aeruginosa* (UNCSAB03-16-02)

Medio	*Forma*	*Elevación*	*Borde*	*Brillo*	*Textura*	*Color*
Agar nutritivo	móvil	Elevada	Regular	Brillante	Cremosa	Transparente

Tabla 36. Características Morfológicas *Pseudomonas aeruginosa* (UNCSAB03-16-03)

Medio	*Forma*	*Elevación*	*Borde*	*Brillo*	*Textura*	*Color*
Agar nutritivo	móvil	plana	Regular	Brillante	Cremosa	Amarillo

Tabla 37. Características Morfológicas *Pseudomonas aeruginosa* (UNCSAB03-20-01)

Medio	*Forma*	*Elevación*	*Borde*	*Brillo*	*Textura*	*Color*
Agar nutritivo	Redonda	Elevada	Regular	Brillante	Cremosa	Amarillo

Tabla 38. Características Morfológicas *Pseudomonas chlororaphis ss aurantiaca* (UNCSAB03-07-05)

Medio	*Forma*	*Elevación*	*Borde*	*Brillo*	*Textura*	*Color*
Agar nutritivo	Irregular	Plana	Irregular	Brillante	Cremosa	Blanco Amarillo

Tabla 39. Características Morfológicas *Pseudomonas marginalis* (UNCSAB03-18-04)

Medio	*Forma*	*Elevación*	*Borde*	*Brillo*	*Textura*	*Color*
Agar nutritivo	irregular	elevada	irregular	Brillante	cremosa	Transparente

Tabla 40. Características Morfológicas *Pseudomonas nitroreducens* (UNCSAB03-21- 06)

Medio	*Forma*	*Elevación*	*Borde*	*Brillo*	*Textura*	*Color*
Agar nutritivo	móvil	Plana	Irregular	Opaca	Cremosa	Blanco

Tabla 41. Características Morfológicas *Pseudomonas tolaasii* (UNCSAB03-16-01)

Medio	*Forma*	*Elevación*	*Borde*	*Brillo*	*Textura*	*Color*
Agar nutritivo	móvil	elevada	regular	brillante	rugosa	Blanco

Tabla 42. Características Morfológicas *Serratia liquefaciens* (UNCSAB03-19-01)

Medio	*Forma*	*Elevación*	*Borde*	*Brillo*	*Textura*	*Color*
Agar nutritivo	Irregular	Elevada	Regular	Brillante	Cremosa	Blanco

Tabla 43. Características Morfológicas *Serratia liquefaciens/grimesii* (UNCSAB03-21-3)

Medio	*Forma*	*Elevación*	*Borde*	*Brillo*	*Textura*	*Color*
Agar nutritivo	Redonda	Elevada	Regular	Brillante	Cremosa	Blanco

Tabla 44. Características Morfológicas *Serratia marcescens ss marcescens* (UNCSAB03-04-01)

Medio	Forma	Elevación	Borde	Brillo	Textura	Color
Agar nutritivo	Irregular	Elevada	Irregular	Opaca	Granulosa	Naranja

Tabla 45. Características Morfológicas *Serratia marcescens ss marcescens* (UNCSAB03-04-05)

Medio	*Forma*	*Elevación*	*Borde*	*Brillo*	*Textura*	*Color*
Agar nutritivo	Irregular	Elevada	Irregular	Brillante	Cremosa	Naranja

Tabla 46. Características Morfológicas *Serratia marcescens ss marcescens* (UNCSAB03-22-01)

Medio	*Forma*	*Elevación*	*Borde*	*Brillo*	*Textura*	*Color*
Agar nutritivo	irregular	Elevada	irregular	brillante	cremosa	Amarillo

Tabla 47. Características Morfológicas *Serratia marcescens ss marcescens* (UNCSAB03-23-01)

Medio	*Forma*	*Elevación*	*Borde*	*Brillo*	*Textura*	*Color*
Agar nutritivo	Redonda	Elevada	Regular	Brillante	Cremosa	Blanco

Tabla 48. Características Morfológicas *Xenorhabdus nematophila* (UNCSAB03-23-04)

Medio	*Forma*	*Elevación*	*Borde*	*Brillo*	*Textura*	*Color*
NBTA	Redonda	Elevada	Regular	Brillante	Cremosa	Azul

Tabla 49. Características Morfológicas *Xenorhabdus nematophila* (UNCSAB03-25-01)

Medio	*Forma*	*Elevación*	*Borde*	*Brillo*	*Textura*	*Color*
NBTA	Redonda	Elevada	Regular	Brillante	Cremosa	Azul

Ejemplo 15. Caracterización de las cepas

Se caracterizó la cepa de los microorganismos empleando el sistema de identificación y caracterización biológica Biolog GEN III microplate®. Con dicho sistema de identificación es posible analizar un microorganismo con 94 pruebas fenotípicas: 71 ensayos de utilización de fuentes de carbono y 23 ensayos de sensibilidad química. El panel provee patrones fenotípicos del microorganismo que pueden ser usadas para identificarlo a nivel de especie. Las Tablas 50 a 58 muestran la Identificación y Caracterización biológica. Se incluyen tanto un control negativo como uno positivo (Tablas 50 a 58).

Tabla 50. Identificación y Caracterización biológica de *Bacillus mojavensis* UNCSAB02-24, *Xenorhabdus nematophila* UNCSAB03-25-01 y *Xenorhabdus* sp. UNCSAB03-23- 04

	Resultado		
	***Bacillus mojavensis* UNCSAB02-24**	***Xenorhabdus nematophila* UNCSAB03-25-01**	***Xenorhabdus* sp. UNCSAB03-23- 04**
Control negativo	-	-	-
Dextrina	+	-	-
D-maltosa	+	-	-
D-trehalosa	+	+/-	+
D-Celobiosa	+	-	-
Gentiobiosa	+	-	-
Sacarosa	+	-	-
D-Turanosa	+/-	-	+/-
Estaquiosa	+/-	-	-
Control positivo	+	+	+
pH 6	+	+	+/-
pH 5	+	-	-
D-Rafinosa	+	-	-
α-D-Lactosa	+	-	-
D-Melibiosa	+/-	+/-	+/-
β-Metil-D-Glucosido	+	+/-	-
D-Salicina	+	-	-
N-Acetil-D-Glucosamina	+	+	+

N-Acetil-β-D- Manosamina	+/-	+/-	+
N-Acetil-D-Galactosamina	-	+/-	+/-
Ácido N-Acetil Neuraminico	-	-	+
NaCl 1%	+	+	-
NaCl 4%	+	-	-
NaCl 8%	+	-	-
α-D-Glucosa	+	+/-	+
D-Manosa	+	+	+
D-Fructosa	+	+	+
D-Galactosa	+/-	+/-	+/-
Metil Glucosa	+/-	+/-	+/-
D-Fucosa	+/-	+/-	+/-
L-Fucosa	+/-	+/-	+/-
L- Ramnosa	+	+/-	+/-
Inosina	+/-	+/-	+/-
Lactato de sodio 1%	+	+/-	+
Ácido Fusidico	-	-	-
D-Serina	-	+	+
D-Sorbitol	+	-	-
D-Manitol	+	-	-
D-Arabitol	+/-	-	-
mio-Inositol	+	+/-	+
Glicerol	+	+	+
D-Glucosa-6-PO_4	+	+	+
D-Fructosa-6-PO_4	+	-	-
Ácido D-Aspártico	+	+	+
D-Serina	-	+	+
Troleandomicina	-	+	+
Rifamicina SV	-	+	+
Minociclina	-	-	-
Gelatina	+	-	-
Glicil-L-Prolina	+	+/-	+/-
L-Alanina	+	+	+
L-Arginina	+	+/-	+/-
L- Ácido Aspartico	+	+	+
L- Acid Glutamico	+	+	+
L-Histidina	+	+	+
L- Ácido Piroglutamico	+/-	+/-	+/-

L-Serina	+	+	+
Lincomicina	-	+	+
Guanidina HCl	+	+/-	+/-
Niaproof 4	-	-	-
Pectina	+	-	-
Acid D-Galacturonico	+	-	-
Ácido L-Galactonico Lactona	+	+/-	+/-
Ácido D-Gluconico	+	+/-	+/-
Ácido D-Glucuronico	+	+/-	+/-
Glucuronamido	+/-	+/-	+/-
Ácido Mucico	+/-	+/-	+/-
Ácido Quinico	+	+/-	+/-
Acid D-Sacarico	+/-	+/-	+/-
Vancomicina	-	+	+
Violeta de Tetrazolio	-	+	+
Azul de Tetrazolio	-	+	+
Ácido p-Hidroxifenilacetico	+/-	-	-
Metil Piruvato	+/-	+	-
Ácido D-Láctico metil éster	+/-	-	+/-
Ácido L-Lactico	+	+	+
Ácido Citrico	+	-	+/-
Ácido α-ceto-Glutarico	+/-	+/-	+/-
Ácido D-Malico	-	+/-	+/-
Ácido L-Malico	+	+/-	+/-
Ácido Bromo-Succinico	+	+/-	+/-
Ácido Nalidixico	+/-	-	-
Cloruro de Litio	+	-	-
Telurito de Potasio	+	+	+
Tween 40	+/-	+	+
Ácido γ-Amino- Butírico	+/-	-	-
Ácido α-Hidroxi- Butírico	-	+/-	+/-
Ácido β-Hidroxi-D,L-Butírico	+/-	-	-
Ácido α-Ceto-Butírico	-	-	-
Ácido Acetoacetico	+	+	+
Ácido Propionico	+/-	-	-
Ácido Acético	+	+	+

Ácido Fórmico	+	-	-
Aztreonam	-	-	+/-
Butirato de Sodio	+	-	-
Bromato de Sodio	-	-	-

Tabla 51. Identificación y Caracterización biológica de *Acinetobacter beijerinckii* UNCSAB03-22-05, *Bacillus thuringiensis/cereus* UNCSAB03-03-06 y *Bacillus amyloliquefaciens ss amyloliquefaciens* UNCSAB03-03-12

Prueba	**Resultado**		
	***Acinetobacter beijerinckii* UNCSAB03-22-05**	***Bacillus thuringiensis/cereus* UNCSAB03-03-06**	***Bacillus amyloliquefaciens ss amyloliquefaciens* UNCSAB03-03-12**
Control negativo	-	-	+/-
Dextrina	-	+	+
D-maltosa	-	+/-	+
D-trehalosa	-	+/-	+
D-Celobiosa	-	-	+
Gentiobiosa	-	-	+
Sacarosa	-	-	+
D-Turanosa	-	-	+/-
Estaquiosa	-	-	+/-
Control positivo	+	+	+
pH 6	+	+	+
pH 5	-	-	+/-
D-Rafinosa	-	-	+
α-D-Lactosa	-	-	+
D-Melibiosa	+/-	-	+/-
β-Metil-D- Glucosido	-	-	+
D-Salicina	-	-	+
N-Acetil-D-Glucosamina	-	+	+
N-Acetil-β-D-Manosamina	-	-	+/-
N-Acetil-D-Galactosamina	-	-	-
Ácido N-Acetil Neuraminico	-	-	-
NaCl 1%	+	+	+
NaCl 4%	-	+	+

NaCl 8%	-	+/-	+
α-D-Glucosa	-	+/-	+
D-Manosa	+/-	-	+
D-Fructosa	-	+/-	+
D-Galactosa	+/-	-	+/-
Metil Glucosa	-	-	+/-
D-Fucosa	-	-	+/-
L-Fucosa	+/-	-	+/-
L- Ramnosa	+/-	-	+/-
Inosina	-	+	+/-
Lactato de Sodio 1%	+	+	+
Ácido Fusidico	-	-	-
D-Serina	+	+	-
D-Sorbitol	-	-	+
D-Manitol	-	-	+
D-Arabitol	-	-	-
mio-Inositol	-	-	-
Glicerol	-	+/-	+
D-Glucosa-6-PO_4	+/-	+	+/-
D-Fructosa-6-PO_4	+/-	+	+/-
Ácido D-Aspártico	-	-	+
D-Serina	+/-	-	-
Troleandomicina	-	-	-
Rifamicina SV	+	-	-
Minociclina	-	-	-
Gelatina	-	+	+
Glicil-L-Prolina	-	-	+
L-Alanina	+	+/-	+
L-Arginina	-	+/-	+
L- Ácido Aspartico	+	+/-	+
L- Acid Glutamico	+	+/-	+
L-Histidina	+	+/-	+
L- Ácido Piroglutamico	-	-	+/-
L-Serina	+	+	+/-
Lincomicina	+	-	-
Guanidina HCl	+/-	+	+
Niaproof 4	+/-	-	-

Pectina	-	-	+
Acid D-Galacturonico	+/-	+	+
Ácido L-Galactonico Lactona	+/-	+/-	-
Ácido D-Gluconico	-	+/-	+
Ácido D-Glucuronico	+/-	+/-	+/-
Glucuronamido	+/-	+/-	+/-
Ácido Mucico	-	-	+/-
Ácido Quinico	-	-	+/-
Acid D-Sacarico	-	-	+/-
Vancomicina	+	-	-
Violeta de Tetrazolio	+	+/-	-
Azul de Tetrazolio	+	-	-
Ácido p-Hidroxifenilacetico	-	-	-
Metil Piruvato	+	+/-	+
Ácido D-Láctico metil éster	-	-	+/-
Ácido L-Lactico	-	+/-	+
Ácido Citrico	+	-	+
Ácido α-ceto- Glutarico	+	-	+/-
Ácido D-Malico	+	-	+
Ácido L-Malico	+	+	+
Ácido Bromo-Succinico	+	-	+/-
Ácido Nalidixico	+/-	-	-
Cloruro de Litio	+/-	+	+
Telurito de Potasio	+	+	+
Tween 40	+	+/-	+
Ácido γ-Amino- Butririco	+	-	+/-
Ácido α-Hidroxi- Butirico	-	-	-
Ácido β-Hidroxi-D,L-Butirico	+/-	-	-
Ácido α-ceto- Butirico	+	-	-
Acid Acetoacetico	+/-	+	+
Acid Propionico	+	-	+/-
Ácido Acetico	+	+/-	+

Acid Formico	+/-	-	+
Aztreonam	+	+	-
Butirato de Sodio	-	+	-
Bromato de Sodio	-	+/-	-

Tabla 52. Identificación y Caracterización biológica de *Bacillus oleronius* UNCSAB03-04-03, *Burkholderia dolosa* UNCSAB03-07-04 y *Pseudomonas aeruginosa* UNCSAB03-16-02

Prueba	**Resultado**		
	***Bacillus oleronius* UNCSAB03-04-03**	***Burkholderia dolosa* UNCSAB03-07-04**	***Pseudomonas aeruginosa* UNCSAB03-16-02**
Control negativo	-	-	-
Dextrina	+	-	+/-
D-maltosa	+/-	-	-
D-trehalosa	+/-	-	-
D-Celobiosa	-	-	-
Gentiobiosa	-	+/-	-
Sacarosa	-	-	-
D-Turanosa	-	-	-
Estaquiosa	-	-	-
Control positivo	+	+	+
pH 6	+	+	+
pH 5	+	+	+/-
D-Rafinosa	-	-	-
α-D-Lactosa	-	-	-
D-Melibiosa	-	+/-	+/-
β-Metil-D-Glucosido	-	-	-
D-Salicina	-	-	-
N-Acetil-D-Glucosamina	-	+/-	+/-
N-Acetil-β-D- Manosamina	-	-	-
N-Acetil-D-Galactosamina	-	-	-
Ácido N-Acetil Neuraminico	-	-	-
NaCl 1%	+	+	+
NaCl 4%	+	+/-	+/-
NaCl 8%	+/-	-	-
α-D-Glucosa	+/-	+	+
D-Manosa	-	+	-

D-Fructosa	-	+	+/-
D-Galactosa	-	+	+/-
Metil Glucosa	-	+/-	+/-
D-Fucosa	-	+	+/-
L-Fucosa	-	+/-	+/-
L- Ramnosa	-	+/-	+/-
Inosina	-	+/-	+/-
Lactato de Sodio 1%	+	+	+
Ácido Fusidico	+/-	+	+
D-Serina	+	+/-	+
D-Sorbitol	-	+	-
D-Manitol	-	+/-	-
D-Arabitol	-	+/-	-
mio-Inositol	-	+/-	-
Glicerol	-	+/-	+/-
D-Glucosa-6-PO_4	-	+/-	+/-
D-Fructosa-6-PO_4	-	+	-
Ácido D-Aspártico	-	-	-
D-Serina	-	-	+/-
Troleandomicina	+/-	+	+
Rifamicina SV	+/-	+	+
Minociclina	+	+/-	+
Gelatina	+/-	+/-	+/-
Glicil-L-Prolina	-	+/-	+/-
L-Alanina	-	+	+/-
L-Arginina	-	+	+
L- Ácido Aspartico	-	+	+/-
L- Acid Glutamico	-	+	+
L-Histidina	-	+	+
L- Ácido Piroglutamico	-	+	+/-
L-Serina	-	+	+/-
Lincomicina	+/-	+	+
Guanidina HCl	+	+/-	+
Niaproof 4	+/-	+	+
Pectina	-	-	+/-
Acid D-Galacturonico	-	+/-	+/-
Ácido L-Galactonico Lactona	-	+/-	-
Ácido D-Gluconico	-	+	+

Ácido D-Glucuronico	-	+/-	-
Glucuronamido	-	+	+
Ácido Mucico	-	+/-	-
Ácido Quinico	-	+	+
Acid D-Sacarico	-	-	-
Vancomicina	+/-	+	+
Violeta de Tetrazolio	+/-	+	+
Azul de Tetrazolio	+/-	+	+
Ácido p-Hidroxifenilacetico	-	+	+
Metil Piruvato	-	+	-
Ácido D-Láctico metil éster	-	+/-	-
Ácido L-Lactico	-	-	+
Ácido Citrico	-	+	+
Ácido α-ceto- Glutarico	-	+	+
Ácido D-Malico	-	+	-
Ácido L-Malico	-	+	+
Ácido Bromo-Succinico	-	+	+/-
Ácido Nalidixico	+/-	+	+
Cloruro de Litio	+	-	-
Telurito de Potasio	+	+	+
Tween 40	-	+	-
Ácido γ-Amino- Butririco	-	+	+
Ácido α-Hidroxi- Butirico	-	+/-	-
Ácido β-Hidroxi-D,L-Butirico	-	+	+
Ácido α-ceto- Butirico	-	+/-	-
Acid Acetoacetico	-	+/-	+/-
Acid Propionico	-	+	+
Ácido Acetico	-	+	+/-
Acid Formico	-	+/-	-
Aztreonam	+	+	+/-
Butirato de Sodio	+	+/-	-
Bromato de Sodio	+/-	-	-

Tabla 53. Identificación y Caracterización biológica de *Pseudomonas aeruginosa* UNCSAB03-16-03, *Pseudomonas marginalis* UNCSAB03-18-04 y *Pseudomonas tolaasii* UNCSAB03-16-01

Prueba	**Resultado**		
	***Pseudomonas aeruginosa* UNCSAB03-16-03**	***Pseudomonas marginalis* UNCSAB03-18-04**	***Pseudomonas tolaasii* UNCSAB03-16-01**
Control negativo	-	-	-
Dextrina	+/-	+/-	+/-
D-maltosa	-	-	-
D-trehalosa	-	+	+
D-Celobiosa	-	-	-
Gentiobiosa	-	-	-
Sacarosa	-	-	-
D-Turanosa	-	-	-
Estaquiosa	-	-	-
Control positivo	+	+	+
pH 6	+	+	+
pH 5	+/-	+	+
D-Rafinosa	-	-	-
α-D-Lactosa	-	-	-
D-Melibiosa	-	-	-
β-Metil-D-Glucosido	-	-	-
D-Salicina	-	-	-
N-Acetil-D-Glucosamina	+/-	+/-	+/-
N-Acetil-β-D-Manosamina	-	-	-
N-Acetil-D-Galactosamina	-	-	-
Ácido N-Acetil Neuraminico	-	-	-
NaCl 1%	+	+	+
NaCl 4%	+/-	+/-	-
NaCl 8%	-	-	-
α-D-Glucosa	+	+	+/-
D-Manosa	+/-	+	+/-
D-Fructosa	+/-	+/-	+/-
D-Galactosa	+/-	+	+
Metil Glucosa	-	-	+/-
D-Fucosa	+/-	+/-	+/-
L-Fucosa	+/-	+/-	+/-
L- Ramnosa	+/-	-	+/-

Inosina	+/-	+/-	+/-
Lactato de Sodio 1%	+	+	+
Ácido Fusidico	+	+	+
D-Serina	+/-	-	-
D-Sorbitol	-	+	-
D-Manitol	+	+	+/-
D-Arabitol	-	+/-	+/-
mio-Inositol	-	+/-	+
Glicerol	+/-	+/-	+/-
D-Glucosa-6-PO_4	+/-	-	-
D-Fructosa-6-PO_4	-	+/-	+/-
Ácido D-Aspártico	-	-	+
D-Serina	+/-	-	-
Troleandomicina	+	+	+
Rifamicina SV	+	+	+
Minociclina	+	-	-
Gelatina	-	+/-	-
Glicil-L-Prolina	+/-	-	-
L-Alanina	+/-	+	+/-
L-Arginina	+	+	+
L- Ácido Aspartico	+	+/-	+/-
L- Acid Glutamico	+	+/-	+
L-Histidina	+	+	+
L- Ácido Piroglutamico	+/-	+/-	+
L-Serina	+/-	+	+
Lincomicina	+	+	+
Guanidina HCl	+/-	+/-	+/-
Niaproof 4	+	+	+
Pectina	-	-	-
Acid D-Galacturonico	+/-	+	+
Ácido L-Galactonico Lactona	+/-	-	+/-
Ácido D-Gluconico	+	+	+
Ácido D-Glucuronico	+/-	+	+
Glucuronamido	-	+/-	+
Ácido Mucico	-	+	+
Ácido Quinico	+	+	+
Acid D-Sacarico	-	+	+

Vancomicina	+	+	+
Violeta de Tetrazolio	+	+	+
Azul de Tetrazolio	+	+	+
Ácido p-Hidroxifenilacetico	+	+	-
Metil Piruvato	+/-	+/-	+/-
Ácido D-Láctico metil éster	-	-	-
Ácido L-Lactico	+	+	+/-
Ácido Citrico	+	+	+
Ácido α-ceto-Glutarico	+	+	+
Ácido D-Malico	-	-	-
Ácido L-Malico	+	+	+
Ácido Bromo-Succinico	+/-	-	+/-
Ácido Nalidixico	+	+/-	-
Cloruro de Litio	-	+/-	+/-
Telurito de Potasio	+	+	+
Tween 40	+	+/-	+/-
Ácido γ-Amino- Butririco	+	+	+/-
Ácido α-Hidroxi- Butirico	-	-	-
Ácido β-Hidroxi-D,L-Butirico	+/-	+/-	+/-
Ácido α-ceto-Butirico	-	-	-
Acid Acetoacetico	+/-	-	-
Acid Propionico	+	+	+/-
Ácido Acetico	+	+	+
Acid Formico	-	-	-
Aztreonam	+	+/-	+/-
Butirato de Sodio	-	-	-
Bromato de Sodio	-	+/-	-

Tabla 54. Identificación y Caracterización biológica de *Brevundimonas diminuta* UNCSAB03-09-04, *Brevundimonas diminuta* UNCSAB03-09-04 y *Escherichia hermannii* UNCSAB03-21-02

Prueba	**Resultado**		
	***Brevundimonas diminuta* UNCSAB03-09-04**	***Delftia tsuruhatensis* UNCSAB03-22-02**	***Escherichia hermannii* UNCSAB03-21-02**

Control negativo	-	-	-
Dextrina	+/-	-	+
D-maltosa	-	-	+
D-trehalosa	-	-	+/-
D-Celobiosa	-	-	+/-
Gentiobiosa	-	-	+/-
Sacarosa	-	-	-
D-Turanosa	-	-	-
Estaquiosa	-	-	-
Control positivo	+	+	+
pH 6	+	+	+
pH 5	-	-	+
D-Rafinosa	-	-	-
α-D-Lactosa	-	-	-
D-Melibiosa	-	-	-
β-Metil-D-Glucosido	-	-	+/-
D-Salicina	-	-	+/-
N-Acetil-D-Glucosamina	-	-	+
N-Acetil-β-D-Manosamina	-	-	+
N-Acetil-D-Galactosamina	-	-	-
Ácido N-Acetil Neuraminico	-	-	+
NaCl 1%	+	+	+
NaCl 4%	-	-	+/-
NaCl 8%	-	-	-
α-D-Glucosa	-	-	+/-
D-Manosa	-	-	+
D-Fructosa	-	+/-	+
D-Galactosa	-	+/-	+
Metil Glucosa	-	-	-
D-Fucosa	-	+/-	+/-
L-Fucosa	-	+/-	+/-
L- Ramnosa	-	+/-	+
Inosina	-	-	+
Lactato de Sodio 1%	+	+	+
Ácido Fusidico	-	-	+/-
D-Serina	+	-	-
D-Sorbitol	-	-	-

D-Manitol	-	+/-	+/-
D-Arabitol	-	-	-
mio-Inositol	-	-	-
Glicerol	-	-	+
D-Glucosa-6-PO_4	+/-	-	+
D-Fructosa-6-PO_4	+/-	-	+
Ácido D-Aspártico	-	+	+/-
D-Serina	-	-	-
Troleandomicina	+/-	+	+
Rifamicina SV	+	+	+
Minociclina	-	-	-
Gelatina	+	-	-
Glicil-L-Prolina	+	-	+
L-Alanina	-	+/-	+
L-Arginina	-	-	-
L- Ácido Aspartico	+	+	+
L- Acid Glutamico	+	+	+
L-Histidina	+	+	+/-
L- Ácido Piroglutamico	-	+/-	+/-
L-Serina	-	-	+
Lincomicina	+	+	+
Guanidina HCl	+/-	+/-	+
Niaproof 4	-	+/-	+
Pectina	-	-	-
Acid D-Galacturonico	-	+/-	+
Ácido L-Galactonico Lactona	+/-	-	-
Ácido D-Gluconico	-	+	+
Ácido D-Glucuronico	+/-	+/-	+
Glucuronamido	+/-	+/-	+/-
Ácido Mucico	-	+	+
Ácido Quinico	-	-	-
Acid D-Sacarico	-	+/-	+
Vancomicina	-	+	+
Violeta de Tetrazolio	+	+	+
Azul de Tetrazolio	+	+	+
Ácido p-Hidroxifenilacetico	-	+	-
Metil Piruvato	-	+/-	+

Ácido D-Láctico metil éster	-	-	+/-
Ácido L-Lactico	-	+/-	+
Ácido Citrico	-	+/-	+/-
Ácido α-ceto-Glutarico	-	+	-
Ácido D-Malico	-	+	-
Ácido L-Malico	-	+	+/-
Ácido Bromo-Succinico	-	+	-
Ácido Nalidixico	+	-	-
Cloruro de Litio	+/-	+/-	+/-
Telurito de Potasio	-	-	+/-
Tween 40	-	+/-	-
Ácido γ-Amino- Butririco	-	-	-
Ácido α-Hidroxi- Butirico	-	-	+/-
Ácido β-Hidroxi-D,L-Butirico	+/-	+/-	-
Ácido α-ceto-Butirico	-	+	-
Acid Acetoacetico	+/-	+/-	+/-
Acid Propionico	-	+	-
Ácido Acetico	+/-	+	+
Acid Formico	-	+/-	-
Aztreonam	+	+/-	+/-
Butirato de Sodio	-	+/-	+/-
Bromato de Sodio	-	-	-

Tabla 55. Identificación y Caracterización biológica de *Pseudomonas aeruginosa* UNCSAB03-13-01, *Pseudomonas aeruginosa* UNCSAB03-20-01 y *Pseudomonas chlororaphis ss aurantiaca* UNCSAB03-07-05

Prueba	**Resultado**		
	***Pseudomonas aeruginosa* UNCSAB03-13-01**	***Pseudomonas aeruginosa* UNCSAB03-20-01**	***Pseudomonas chlororaphis ss aurantiaca* UNCSAB03-07-05**
Control negativo	-	-	-
Dextrina	+/-	+/-	+/-
D-maltosa	-	-	-
D-trehalosa	-	+/-	+
D-Celobiosa	-	-	-
Gentiobiosa	-	-	-

Sacarosa	-	-	-
D-Turanosa	-	-	-
Estaquiosa	-	-	-
Control positivo	+	+	+
pH 6	+	+	+
pH 5	+	+	+
D-Rafinosa	-	-	-
α-D-Lactosa	-	-	-
D-Melibiosa	-	-	-
β-Metil-D-Glucosido	-	-	-
D-Salicina	-	-	-
N-Acetil-D-Glucosamina	+/-	-	+/-
N-Acetil-β-D- Manosamina	-	-	-
N-Acetil-D-Galactosamina	-	-	-
Ácido N-Acetil Neuraminico	-	-	-
NaCl 1%	+	+	+
NaCl 4%	+/-	+/-	+/-
NaCl 8%	-	-	-
α-D-Glucosa	+	+	+
D-Manosa	+/-	-	+/-
D-Fructosa	+/-	+/-	+
D-Galactosa	+/-	+/-	+/-
Metil Glucosa	-	-	-
D-Fucosa	+/-	+/-	+/-
L-Fucosa	+/-	+/-	+/-
L- Ramnosa	+/-	+/-	+/-
Inosina	+/-	+/-	+
Lactato de Sodio 1%	+	+	+
Ácido Fusidico	+	+	+
D-Serina	+/-	+/-	+/-
D-Sorbitol	-	-	-
D-Manitol	+	+/-	+
D-Arabitol	+/-	-	+/-
mio-Inositol	+/-	-	+
Glicerol	+	+/-	+
D-Glucosa-6-PO_4	+/-	-	-
D-Fructosa-6-PO_4	-	-	-

Ácido D-Aspártico	-	-	-
D-Serina	-	+/-	+
Troleandomicina	+	+	+
Rifamicina SV	+	+	+
Minociclina	+	+	+
Gelatina	-	-	-
Glicil-L-Prolina	+	+	-
L-Alanina	+/-	+/-	+
L-Arginina	+	+	+
L- Ácido Aspartico	+	+	+
L- Acid Glutamico	+	+	+
L-Histidina	+	+	+
L- Ácido Piroglutamico	+	+	+
L-Serina	+/-	+/-	+
Lincomicina	+	+	+
Guanidina HCl	+/-	+/-	+/-
Niaproof 4	+	+	+
Pectina	-	-	-
Acid D-Galacturonico	+/-	-	+/-
Ácido L-Galactonico	+/-	+/-	+/-
Lactona			
Ácido D-Gluconico	+	+	+
Ácido D-Glucuronico	+/-	+/-	+/-
Glucuronamido	+	-	+/-
Ácido Mucico	-	-	+
Ácido Quinico	+	+	+
Acid D-Sacarico	-	-	+
Vancomicina	+	+	+
Violeta de Tetrazolio	+	+	+
Azul de Tetrazolio	+	+	+
Ácido p-Hidroxifenilacetico	+	+	+
Metil Piruvato	+/-	+/-	+/-
Ácido D-Láctico metil éster	-	-	-
Ácido L-Lactico	+	+/-	+
Ácido Citrico	+	+	+
Ácido α-ceto-Glutarico	+	+/-	+
Ácido D-Malico	-	-	-
Ácido L-Malico	+	+	+

Ácido Bromo-Succinico	+/-	-	-
Ácido Nalidixico	+	+	+
Cloruro de Litio	-	-	+
Telurito de Potasio	+	+	+
Tween 40	+/-	+/-	+/-
Ácido γ-Amino- Butririco	+	+	+
Ácido α-Hidroxi- Butirico	-	-	-
Ácido β-Hidroxi-D,L-Butirico	+/-	+/-	+/-
Ácido α-ceto-Butirico	-	-	-
Acid Acetoacetico	+/-	-	+/-
Acid Propionico	+	+	+
Ácido Acetico	+	+/-	+
Acid Formico	-	-	-
Aztreonam	+/-	+	+/-
Butirato de Sodio	-	-	-
Bromato de Sodio	+/-	+/-	+/-

Tabla 56. Identificación y Caracterización biológica de *Pseudomonas nitroreducens* UNCSAB03-21-06, *Serratia liquefaciens* UNCSAB03-19-01 y *Serratia liquefaciens/ grimesii* UNCSAB03-21-03

Prueba	**Resultado**		
	***Pseudomonas nitroreducens* UNCSAB03-21-06**	***Serratia liquefaciens* UNCSAB03-19-01**	***Serratia liquefaciens/ grimesii* UNCSAB03-21-03**
Control negativo	-	-	-
Dextrina	-	+/-	+/-
D-maltosa	-	+/-	+/-
D-trehalosa	-	+	+/-
D-Celobiosa	-	+/-	+/-
Gentiobiosa	-	+/-	+/-
Sacarosa	-	+	+/-
D-Turanosa	-	+	+
Estaquiosa	-	+	+
Control positivo	+	+	+
pH 6	+	+	+
pH 5	+	+	+/-
D-Rafinosa	-	+	+

α-D-Lactosa	-	+/-	+/-
D-Melibiosa	-	+/-	+/-
β-Metil-D-Glucosido	-	+	+/-
D-Salicina	-	+	+
N-Acetil-D-Glucosamina	-	+	+
N-Acetil-β-D- Manosamina	-	+	+
N-Acetil-D-Galactosamina	-	+	+/-
Ácido N-Acetil Neuraminico	-	+/-	+/-
NaCl 1%	+	+	+
NaCl 4%	+/-	+/-	+/-
NaCl 8%	-	-	-
α-D-Glucosa	+	+	+/-
D-Manosa	-	+	+/-
D-Fructosa	+/-	+	+/-
D-Galactosa	+/-	+	+
Metil Glucosa	-	+	+
D-Fucosa	+/-	+	+
L-Fucosa	+/-	+	+
L- Ramnosa	+/-	+	+/-
Inosina	-	+/-	+/-
Lactato de Sodio 1%	+	+	+
Ácido Fusidico	+	+	+/-
D-Serina	+/-	+	+
D-Sorbitol	-	+/-	+/-
D-Manitol	-	+	+/-
D-Arabitol	-	+/-	-
mio-Inositol	-	+	+
Glicerol	+/-	+	+/-
D-Glucosa-6-PO_4	-	+	+
D-Fructosa-6-PO_4	-	+	+
Ácido D-Aspártico	-	+	+/-
D-Serina	-	+	+
Troleandomicina	+	+	+
Rifamicina SV	+	+	+
Minociclina	+	+/-	+/-
Gelatina	-	+	+
Glicil-L-Prolina	-	+	+

L-Alanina	+/-	+	+
L-Arginina	+	+/-	+/-
L- Ácido Aspartico	+	+	+
L- Acid Glutamico	+	+	+
L-Histidina	-	+	+
L- Ácido Piroglutamico	-	+/-	+/-
L-Serina	+/-	+	+
Lincomicina	+	+	+
Guanidina HCl	+/-	+/-	+
Niaproof 4	+	+	+
Pectina	-	+	+/-
Acid D-Galacturonico	+/-	+	+
Ácido L-Galactonico	+/-	+	+
Lactona			
Ácido D-Gluconico	+	+	+
Ácido D-Glucuronico	+/-	+	+
Glucuronamido	+/-	+	+
Ácido Mucico	-	+/-	+/-
Ácido Quinico	+	+/-	-
Acid D-Sacarico	-	+/-	+/-
Vancomicina	+	+	+
Violeta de Tetrazolio	+	+	+
Azul de Tetrazolio	+	+	+
Ácido p-Hidroxifenilacetico	-	+/-	+/-
Metil Piruvato	+	+/-	+/-
Ácido D-Láctico metil éster	-	-	+/-
Ácido L-Lactico	+	+	+
Ácido Citrico	+	+	+
Ácido α-ceto-Glutarico	+	+	+
Ácido D-Malico	-	+	+
Ácido L-Malico	+	+	+
Ácido Bromo-Succinico	+/-	+/-	+/-
Ácido Nalidixico	+	-	-
Cloruro de Litio	-	+/-	+/-
Telurito de Potasio	+	-	-
Tween 40	+/-	+/-	+
Ácido γ-Amino- Butririco	+	-	-

Ácido α-Hidroxi- Butirico	-	+/-	+/-
Ácido β-Hidroxi-D,L-Butirico	+	-	-
Ácido α-ceto-Butirico	-	-	-
Acid Acetoacetico	-	+/-	-
Acid Propionico	+	+/-	-
Ácido Acetico	+	+	+/-
Acid Formico	+/-	+/-	+/-
Aztreonam	+/-	+/-	+/-
Butirato de Sodio	+/-	+/-	+/-
Bromato de Sodio	-	-	-

Tabla 57. Identificación y Caracterización biológica de *Serratia marcescens ss marcescens* UNCSAB03-04-01, *Serratia marcescens ss marcescens* UNCSAB03-04-05 y *Serratia marcescens ss marcescens* UNCSAB03-23-01

Prueba	**Resultado**		
	***Serratia marcescens ss marcescens* UNCSAB03-04-01**	***Serratia marcescens ss marcescens* UNCSAB03-04-05**	***Serratia marcescens ss marcescens* UNCSAB03-23-01**
Control negativo	-	-	-
Dextrina	+/-	+/-	+/-
D-maltosa	+	+	+
D-trehalosa	+	+	+
D-Celobiosa	+/-	+/-	+/-
Gentiobiosa	+/-	+/-	+/-
Sacarosa	-	+	+
D-Turanosa	-	-	-
Estaquiosa	-	-	-
Control positivo	+	+	+
pH 6	+	+	+
pH 5	+/-	+/-	+
D-Rafinosa	-	+/-	-
α-D-Lactosa	+/-	+/-	-
D-Melibiosa	+/-	+/-	+/-
β-Metil-D-Glucosido	+	+	+
D-Salicina	+	+	+
N-Acetil-D-	+	+	+

Glucosamina			
N-Acetil-β-D- Manosamina	+/-	+/-	+
N-Acetil-D-Galactosamina	+	+	+
Ácido N-Acetil Neuraminico	+/-	-	-
NaCl 1%	+	+	+
NaCl 4%	+	+/-	+/-
NaCl 8%	+/-	-	-
α-D-Glucosa	+/-	+	+
D-Manosa	+	+	+
D-Fructosa	+	+	+
D-Galactosa	+	+	+
Metil Glucosa	+/-	+	+/-
D-Fucosa	+	+	+
L-Fucosa	+	+/-	+
L- Ramnosa	+/-	+/-	+/-
Inosina	+	+	+
Lactato de Sodio 1%	+	+	+
Ácido Fusidico	+	+	+/-
D-Serina	+	+/-	+
D-Sorbitol	+	+	+
D-Manitol	+	+	+
D-Arabitol	+/-	+	+/-
mio-Inositol	+	+	+
Glicerol	+	+	+
D-Glucosa-6-PO_4	+	+	+
D-Fructosa-6-PO_4	+	+	+
Ácido D-Aspártico	+/-	+/-	+/-
D-Serina	+	+	+
Troleandomicina	+	+	+
Rifamicina SV	+	+	+
Minociclina	+	+/-	+
Gelatina	+	+	+
Glicil-L-Prolina	+	+	+
L-Alanina	+	+	+
L-Arginina	+/-	+/-	+/-
L- Ácido Aspartico	+	+	+

L- Acid Glutamico	+	+	+
L-Histidina	+	+	+
L- Ácido Piroglutamico	+/-	+/-	-
L-Serina	+	+	+
Lincomicina	+	+	+
Guanidina HCl	+	+	+
Niaproof 4	+	+	+
Pectina	+	+	+/-
Acid D-Galacturonico	+	+/-	+
Ácido L-Galactonico Lactona	+/-	+/-	-
Ácido D-Gluconico	+	+	+
Ácido D-Glucuronico	+	+/-	+
Glucuronamido	+/-	+	+
Ácido Mucico	-	+/-	-
Ácido Quinico	-	-	-
Acid D-Sacarico	+/-	+/-	-
Vancomicina	+	+	+
Violeta de Tetrazolio	+	+	+
Azul de Tetrazolio	+	+	+
Ácido p-Hidroxifenilacetico	+	+	+
Metil Piruvato	+/-	+	+/-
Ácido D-Láctico metil éster	-	+/-	-
Ácido L-Lactico	+	+	+
Ácido Citrico	+	+	+
Ácido α-ceto-Glutarico	+	+	+/-
Ácido D-Malico	+/-	+	+/-
Ácido L-Malico	+	+	+
Ácido Bromo-Succinico	+/-	+/-	+/-
Ácido Nalidixico	-	-	-
Cloruro de Litio	+	+/-	+
Telurito de Potasio	-	-	-
Tween 40	+/-	+/-	+/-
Ácido γ-Amino- Butririco	+/-	+/-	+/-
Ácido α-Hidroxi- Butirico	+/-	+	-
Ácido β-Hidroxi-D,L-Butirico	+	+	+/-
Ácido α-ceto-Butirico	-	-	-

Acid Acetoacetico	+/-	+/-	+
Acid Propionico	-	+	+
Ácido Acetico	+	+/-	+/-
Acid Formico	+/-	+/-	+/-
Aztreonam	+/-	+/-	+/-
Butirato de Sodio	+/-	+/-	+/-
Bromato de Sodio	-	-	-

Tabla 58. Identificación y Caracterización biológica de *Xenorhabdus nematophila* UNCSAB03-23-04, *Xenorhabdus nematophila* UNCSAB03-25-01, *Serratia marcescens ss marcescens* UNCSAB03-22-01

Prueba	**Resultado**		
	***Xenorhabdus nematophila* UNCSAB03-23-04**	***Xenorhabdus nematophila* UNCSAB03-25-01**	***Serratia marcescens ss marcescens* UNCSAB03-22-01**
Control negativo	-	-	-
Dextrina	-	-	+/-
D-maltosa	-	-	+/-
D-trehalosa	+	+/-	+
D-Celobiosa	-	-	-
Gentiobiosa	-	-	+/-
Sacarosa	-	-	+
D-Turanosa	+/-	-	-
Estaquiosa	-	-	-
Control positivo	+	+	+
pH 6	+/-	+	+
pH 5	-	-	+
D-Rafinosa	-	-	-
α-D-Lactosa	-	-	+/-
D-Melibiosa	+/-	+/-	+/-
β-Metil-D-Glucosido	-	+/-	+/-
D-Salicina	-	-	+
N-Acetil-D-Glucosamina	+	+	+
N-Acetil-β-D- Manosamina	-	-	+
N-Acetil-D-Galactosamina	+/-	+/-	+
Ácido N-Acetil Neuraminico	-	-	-

NaCl 1%	+	+	+
NaCl 4%	-	-	+
NaCl 8%	-	-	+/-
α-D-Glucosa	+	+/-	+
D-Manosa	+	+	+/-
D-Fructosa	+	+	+/-
D-Galactosa	+/-	+/-	+
Metil Glucosa	+/-	+/-	+/-
D-Fucosa	+/-	+/-	+
L-Fucosa	+/-	+/-	+
L- Ramnosa	+/-	-	+/-
Inosina	+/-	+/-	+
Lactato de Sodio 1%	+	+/-	+
Ácido Fusidico	-	-	+/-
D-Serina	+	+	+
D-Sorbitol	-	-	+/-
D-Manitol	-	-	+/-
D-Arabitol	-	-	+/-
mio-Inositol	+	+/-	+
Glicerol	+	+	+/-
D-Glucosa-6-PO_4	+	+	+
D-Fructosa-6-PO_4	+	+	+
Ácido D-Aspártico	+	+	+/-
D-Serina	-	-	+
Troleandomicina	+	+	+
Rifamicina SV	+	+	+
Minociclina	-	-	+
Gelatina	-	-	+
Glicil-L-Prolina	+/-	+/-	+
L-Alanina	+	+	+
L-Arginina	+/-	+/-	+/-
L- Ácido Aspartico	+	+	+
L- Acid Glutamico	+	+	+
L-Histidina	-	+	+
L- Ácido Piroglutamico	+/-	+/-	-
L-Serina	+	+	+
Lincomicina	+	+	+
Guanidina HCl	+/-	+/-	+

Niaproof 4	-	-	+
Pectina	-	-	+
Acid D-Galacturonico	-	-	+
Ácido L-Galactonico Lactona	+/-	+/-	-
Ácido D-Gluconico	+/-	+/-	+
Ácido D-Glucuronico	+/-	+/-	+
Glucuronamido	+/-	+/-	+
Ácido Mucico	+/-	+/-	-
Ácido Quinico	+/-	+/-	-
Acid D-Sacarico	+/-	-	-
Vancomicina	+	+	+
Violeta de Tetrazolio	+	+	+
Azul de Tetrazolio	+	+	+
Ácido p-Hidroxifenilacetico	-	-	+
Metil Piruvato	+	+	+/-
Ácido D-Láctico metil éster	-	-	-
Ácido L-Lactico	+	+	+
Ácido Citrico	+/-	-	+
Ácido α-ceto-Glutarico	+/-	+/-	+/-
Ácido D-Malico	+/-	+/-	+/-
Ácido L-Malico	-	+/-	+
Ácido Bromo-Succinico	+/-	+/-	-
Ácido Nalidixico	-	-	-
Cloruro de Litio	-	-	+
Telurito de Potasio	+	+	-
Tween 40	+	+	+/-
Ácido γ-Amino- Butririco	-	-	+
Ácido α-Hidroxi- Butirico	+/-	+/-	+/-
Ácido β-Hidroxi-D,L-Butirico	-	-	+/-
Ácido α-ceto-Butirico	+	+	-
Acid Acetoacetico	+	+	+
Acid Propionico	-	+	-
Ácido Acetico	+	+	+/-
Acid Formico	-	-	+/-
Aztreonam	+/-	-	+/-
Butirato de Sodio	-	-	+/-

Bromato de Sodio	-	-	-

A guide to solid phase extraction

Introduction to solid phase extraction

Solid phase extraction (SPE) is the most powerful technique currently available for rapid, selective sample preparation. The versatility of SPE allows it to be used for a number of purposes, such as purification, trace enrichment, solvent exchange (analytes are transferred from one particular matrix environment into another, e.g. aqueous to organic), desalting, derivatisation (analytes are retained on a sorbent, derivatised, then eluted) and class fractionation (a sample is separated into different compound groups which share common properties).

SPE offers many benefits and advantages over more traditional sample preparation techniques (such as liquid-liquid extraction), including the following:

1. *High recoveries of the analytes*
2. *Concentration of the analytes*
3. *Highly purified extracts*
4. *Ability to simultaneously extract analytes of wide polarity range*
5. *Ease of automation*
6. *Compatibility with instrumental analysis*
7. *Reduction in organic solvent consumption*

As a result of these various benefits, SPE usage has grown dramatically over the last fifteen years, and continues to grow as the preferred technique for state-of-the-art sample preparation.

SPE is a very simple technique to use, employing inexpensive, disposable extraction columns that are available in a multitude of column sizes and sorbents (pages 17-49). High throughput 96-well SPE formats are also available (pages 67-76).

In principle, SPE is analogous to liquid-liquid extraction. As a liquid sample is passed through the SPE column, compounds are 'extracted' from the sample onto the sorbent material in the column. Interferences can then be selectively removed from the column through the correct choice of 'wash' solvents. Finally, the desired analytes may be selectively recovered from the column by an elution solvent, resulting in a highly purified extract. This extract is often significantly more concentrated than the original sample. Alternatively, an extraction column may be selected which retains the interferences in the sample, but allows the analytes to pass through unretained. Examples of all the different modes of SPE are illustrated schematically on pages 123-128.

Most ISOLUTE SPE columns contain sorbents with an average particle size of 50 µm. Most organic solvents will flow through the columns under gravity, but for aqueous and other viscous samples and solvents, liquids must be passed through the columns using one of three methods:

1. *Vacuum applied to the column outlet*
2. *Pressure applied to the column inlet*
3. *Centrifugation*

Of these methods, vacuum is the most commonly employed. This catalogue includes a section on the VacMaster vacuum manifolds and accessories specifically designed for simultaneously processing multiple extraction columns (page 77) and plates (pages 73-76). The use of pressure applied to the column inlet is applicable to single sample manual processing as well as to full automation of the SPE procedures. The syringe adaptors for single sample processing are listed on page 52 of this catalogue. Information on the automation of SPE column processing is provided on pages 112-113.

The properties of SPE sorbents

Sorbents for solid phase extraction

Solid phase extraction is performed using either silica based or organic resin based sorbents, with suitable physical characteristics and chemical properties. The nature of the base material, and the additional functional groups both affect the way that the sorbents are used.

Sorbent base material

The sorbents in all cases (whether they are called "silica based" or organic resins) are three dimensional polymeric materials which are manufactured under conditions designed to provide a very porous but rigid material with a high surface area. There are two approaches to the manufacturing of such materials: 1) the formation of a "gel" which is then crushed to provide irregularly shaped solid particles of a size appropriate for use in SPE; or 2) formation of individual particles in a rapidly stirred reactor which produces particles which are both spherical and of an appropriate size for SPE.

For example, ISOLUTE Silica is manufactured as a gel, crushed and sieved, and therefore is irregular in shape, whereas ISOLUTE 101 is manufactured as spherical particles. The physical properties of the material (pore size, rigidity and surface area) are carefully controlled during the manufacturing process to ensure that the particles have uniform, reproducible characteristics.

Physical properties of SPE sorbents

Particle size distribution. The typical mean particle size for solid phase extraction sorbents is around 50 µm. However, the distribution of particle size around this mean impacts on the performance of the SPE column at every stage of the SPE process. The ideal particle size distribution is a narrow Gaussian curve (see fig 1) which ensures even flow characteristics of solvents and sample through the column. Presence of significant levels of fines can lead to sorbent and analyte breakthrough, incomplete recoveries, and the use of excessive solvent volumes (fig 2).

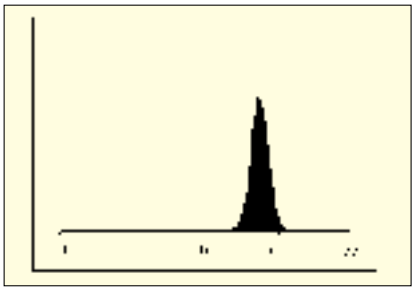

Fig. 1 Ideal particle size distribution for SPE

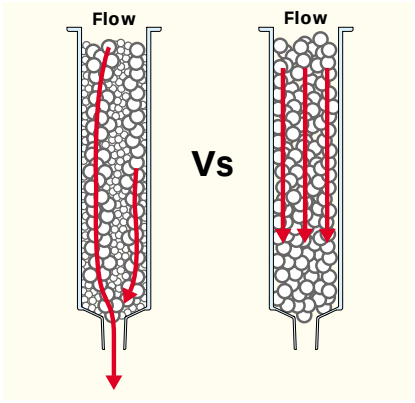

Fig. 2 Impact of fines on flow

Surface area. During the extraction process, the surface of the sorbent must be in contact with the sample. The efficiency of the extraction is increased when this interface (the surface area of the sorbent) is large. The size of the sorbent particles, and the porous nature of the base sorbent lead to a high surface area suitable for SPE. Both the surface area and pore size of the sorbents must be carefully controlled to give reproducible SPE results. In general, the higher the surface area of the base material, the smaller the sorbent bed needed for efficient extraction.

For example, silica based sorbents are typically 500 m^2 / g, ENV+ 1100 m^2 / g.

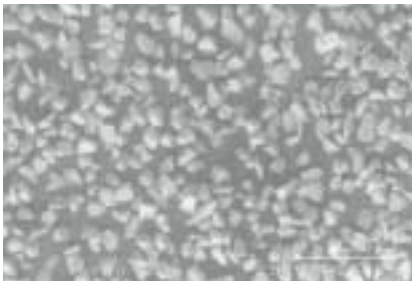

Fig. 3 Irregular silica particles

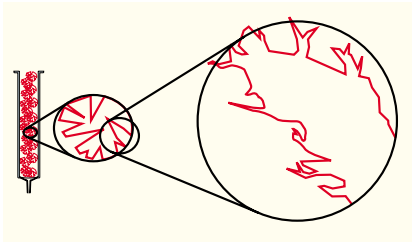

Fig. 4 Porous nature of sorbent

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Rigidity: SPE sorbents are packed into columns with fixed bed dimensions. In order to maintain suitable flow conditions for solvents and samples during the extraction process, the sorbent must not shrink or swell when exposed to solvents. Silica particles are rigid and sorbents based on silica particles are very solvent resistant. Traditionally, resin based sorbents can be affected by differing solvent environments, but today's 'third' generation of highly cross-linked resins overcome this and are suitable for SPE.

Chemical characteristics of SPE sorbents

Base material: Resin
Depending on the monomers used to build the polymeric resin, and the processes used during polymerisation, many resins can be used for solid phase extraction without further processing. These resins are hydrophobic and offer a single, extremely non-selective retention mechanism.

Surface wettability: Some resins, such as ISOLUTE ENV+, have a specially adapted surface to make them 'wettable' or hydrophilic, eliminating the need for a conditioning step when extracting aqueous samples.

To further increase the applicability of resins, the surface can be modified by introducing acidic or basic functional groups. The resulting materials offer both hydrophobic (due to the polymer 'backbone') and ion exchange retention mechanisms, and can be used as 'mixed-mode' sorbents.

Base material: Silica
The surface of silica particles is heterogeneous, with a variety of different types of silanol groups present. Silica can be used as an SPE sorbent without further modification. However, to increase it's applicability, and the options available to the scientist for choosing the appropriate extraction mechanism, the surface of the silica material is usually modified by bonding a wide variety of functional groups to the surface. The nature of the functionality can be non-polar (e.g. C18), polar (e.g. NH2), ionic (e.g. propylsulphonic acid) or mixed-mode (e.g. C8 / cation exchange).

In general, all resins have significant hydrophobic character due to the nature of the polymer backbone, whereas the hydrophobic nature of silica based sorbents is entirely dependant on the bonded groups.

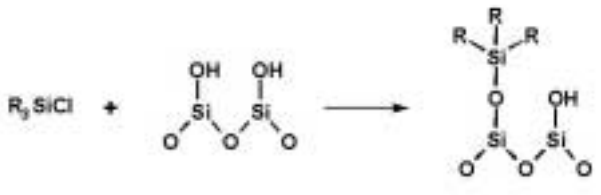
How is the silica bonded?

Bonded silica sorbents are manufactured by reacting an organosilane with the silica surface. The organosilanes that are used consist of a silicon atom bonded to an organic functional group like C18, and 1 - 3 chlorine atoms (depending on the type of bonding that is desired). The two common types of bonding are monofunctional, where the organosilane has one chlorine atom, and trifunctional, where the organosilane has three chlorine atoms. Monofunctional chemistry yields a product having a lightly loaded surface, and therefore more silanol groups than trifunctionally bonded silicas. The silanol groups are very accessible and for this reason the polar character of sorbents manufactured using monofunctional silanes (e.g. ISOLUTE MF C18) can be very useful.

Sorbents manufactured using monofunctional silanes tend to be less stable to extremes of pH because of the single point of attachment of the silane to the silica particle. Trifunctional bonding chemistry gives rise to a polymeric surface, having a higher carbon loading and fewer silanol groups.

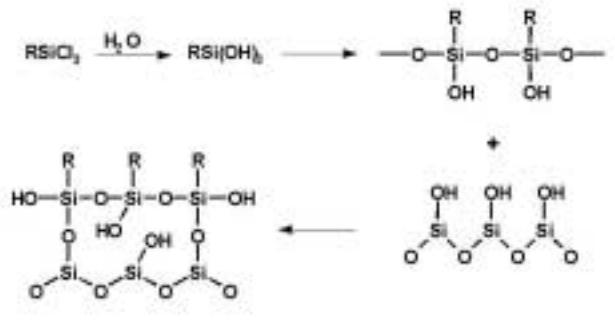
The chemistry of modified silica SPE sorbents

Monochlorosilane chemistry



Compared to monofunctional silane sorbents, trifunctional silane based sorbents are significantly more stable to pH extremes. The multi-point attachment of polymeric silanes to the silica surface slows down the hydrolysis of the silanes. Accessibility of analytes to the polar silanol groups is reduced compared to the monofunctional silane bonded sorbents. Therefore the result of the bonding process is a very heterogeneous surface.

Trichlorosilane chemistry

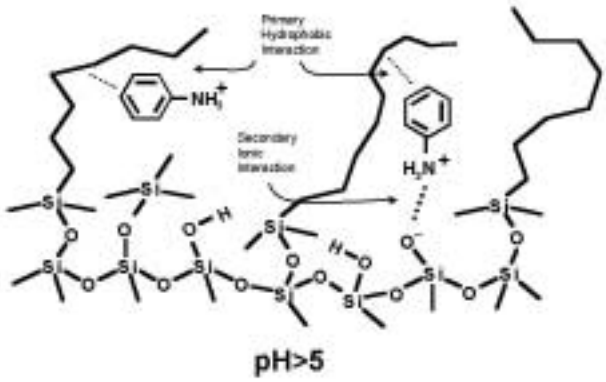


What assurance is there that the product performance will not change?

By carefully controlling the chemistry during the bonding process, IST produces sorbents that are extremely uniform from batch to batch. To ensure batch to batch consistency, many quality control tests are performed. A number of these are listed on page 10.

IST manufactures a wide variety of ISOLUTE sorbents using these processes. They are listed on pages 8-9.

Interactions on ISOLUTE C8



Endcapping: what is it and why do it?

Reacting the bonded silica with trimethyl silane can reduce the number of silanol groups even further. This process is known as endcapping. Fewer silanol groups results in a reduction of polar and ionic secondary interactions associated with the surface. These are the interactions that accompany the primary (hydrophobic) interaction.
For many applications, the presence of residual silanol groups is very beneficial to the extraction process. They provide secondary interactions with analytes, enhancing extraction effectiveness, as well as facilitating the use of more aggressive interference elution washes. Where this is the case, the non-endcapped versions of the non-polar sorbents should always be used to ensure method robustness. Request Technical Note TN112 for further information.

Due to steric factors, even with endcapping, it is impossible to react all of the silanol groups, and the result is a surface that is still quite heterogeneous. Most of the ISOLUTE non-polar phases are available in both the endcapped and non-endcapped forms.

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ISOLUTE sorbent retention mechanisms

ISOLUTE SPE columns are available in a wide selection of sorbent chemistries (Table 1). The chemistry behind these sorbents is discussed on pages 115-118 of this guide. Each of the ISOLUTE chemistries exhibits unique properties for retention of analytes through a variety of molecular interactions (often referred to as 'mechanisms') between the analytes and sorbent. The most common retention mechanisms in SPE are based on Van der Waals forces ('non-polar' interactions), hydrogen bonding and dipole-dipole forces ('polar' interactions) and cation - anion interactions ('ionic' interactions). Each sorbent offers a unique mix of these properties which can be applied to a wide variety of extraction problems. The properties of each sorbent are fully described on the column ordering page of each ISOLUTE chemistry (pages 18-49).

Table 1. ISOLUTE sorbents and retention mechanisms

Primary retention mechanism of ISOLUTE SPE columns / sorbents			
Non-polar	**Mixed-mode (Non-polar / ion exchange)**	**Ion exchange**	**Polar**
Matrix: aqueous	**Matrix: aqueous**	**Matrix: aqueous**	**Matrix: non-polar**
ENV+ pages 18-19	Confirm HCX pages 36-37 (C8 / cation exchange)	NH2 page 39	CN page 33
101 page 20	HCX-3 pages 36-37 (C18 / cation exchange)	PSA page 40	Silica page 46
C18 page 21	HCX-5 pages 36-37 (C4 / cation exchange)	SAX page 41	DIOL page 47
C18(EC) * page 22	Confirm HAX page 35 (C8 anion exchange)	PE-AX page 42	NH2 page 39
MF C18 ** page 23		CBA page 42	PSA page 40
C8 page 24		SCX page 45	Also FL, AL-A, AL-B, AL-N
C8(EC) * page 25		SCX-2 page 45	**Mixed phase**
C6 page 26		SCX-3 page 45	**Matrix: non-polar**
C4 page 27			PSA / SAX pages 40-41 (polar / weak ion-exchange)
C2 page 28			NH2 / SAX pages 39-41 (polar / weak ion-exchange)
C2(EC) * page 29			
CH(EC) * page 30			
PH page 31			
PH(EC) * page 32			
CN page 33			
CN(EC) * page 34			

** (EC) = Endcapped.*

*** MF C18: A C18 sorbent manufactured using a monofunctional silane.*

Information on the significance of 'endcapping' and monofunctional silane chemistry can be found in the section on 'The chemistry of modified silica sorbents', page 118.

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A guide to solid phase extraction

This extensive range of chemistries facilitates one of the most powerful aspects of SPE - high selectivity. The selectivity of an extraction technique is its ability to separate the analyte of interest from interferences in the sample matrix. The highly selective nature of SPE is due to two primary factors. First, each available extraction sorbent chemistry offers unique and distinctive retention properties that can be exploited to address a wide range of analyte characteristics. The second factor is best understood by comparison with liquid-liquid extraction. In liquid-liquid extraction, the two liquids (phases) involved must be immiscible. (Clearly, an aqueous sample cannot be extracted directly with methanol). In SPE however, one phase is a solid sorbent, and therefore is by definition 'immiscible' with any extraction solvent used. This results in a huge variety of possible sorbent / solvent combinations to effect highly selective extractions. Combine this with the choice of SPE operating modes outlined in this guide, and the scope for SPE to solve many of the most demanding sample preparation problems can be readily appreciated.

Guide to sorbent selection

The correct choice of SPE column is critical to ensure a successful SPE extraction procedure. When considering a specific extraction problem, many different aspects influence column selection, including:

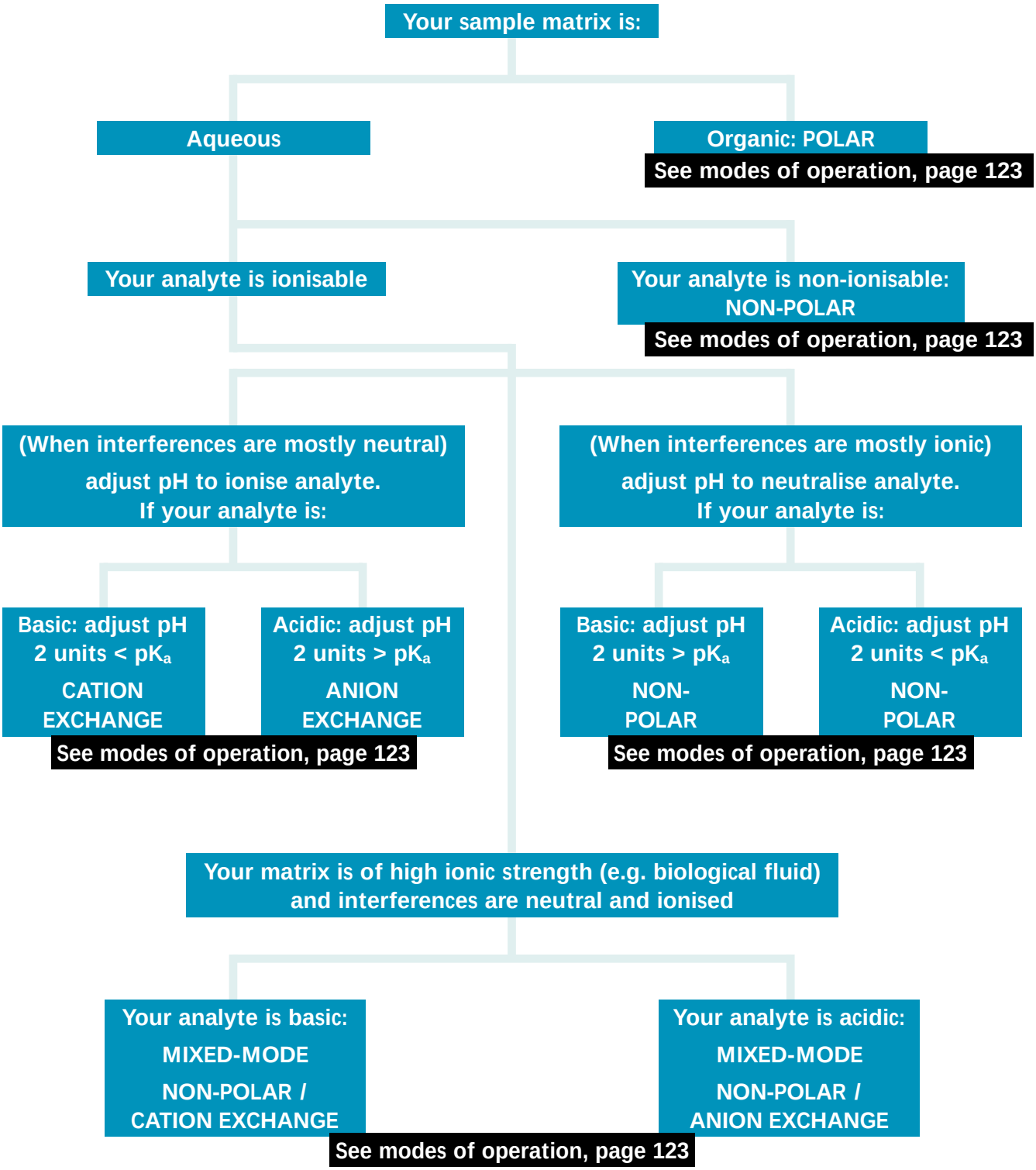
a. *Nature of the analytes*
b. *Nature of the sample matrix*
c. *Degree of purity required*
d. *Nature of major contaminants in the sample*
e. *Final analytical procedure*

In general, rather than evaluating a single column or bonded phase for suitability to a given extraction, it is recommended that a variety of phases be 'screened' for analyte retention simultaneously. Phases are typically grouped in four general categories for purposes of screening, based on the principal retention mechanisms of the phases, non-polar, mixed-mode, polar and ion exchange. To accelerate the SPE method development process, IST provides a number of method development kits based on these four modes of extraction (pages 15-16). These kits allow the scientist to screen for analyte retention / elution characteristics on a number of different sorbents that share the same retention mechanism.

A guide to solid phase extraction

Selecting a mechanism

The guide on this page briefly outlines the decision making process required to choose a suitable extraction mechanism.



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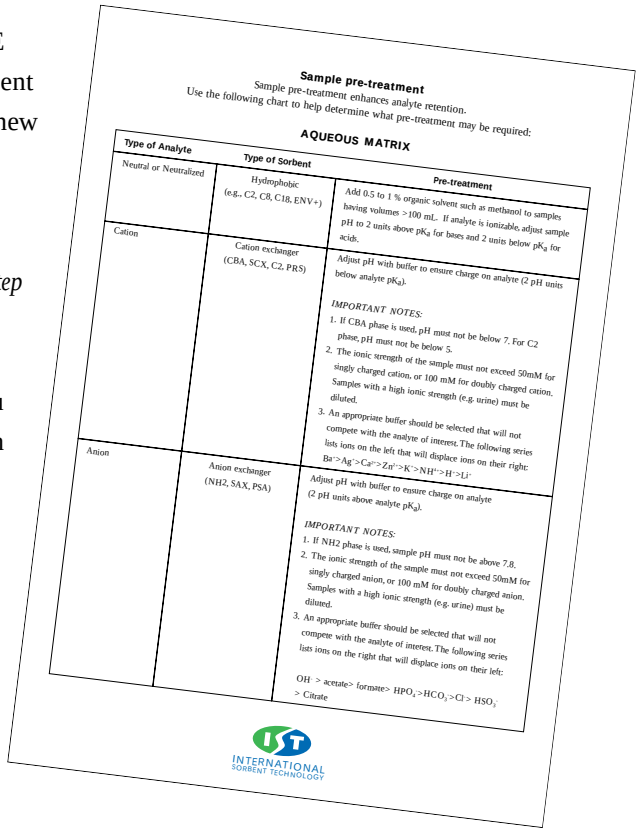
A guide to solid phase extraction

The primary factor influencing the choice of sorbent group for screening is based on the properties of the analyte, (i.e. the functional groups on the analyte promoting interaction or retention of the analyte on the respective bonded phase). The chemical structure of the analyte(s) of interest will indicate which group of sorbents to screen. Analyte functional groups should be correlated with sorbent retention mechanisms, and whether the matrix is aqueous or organic solvent based.

The choice of sorbents is influenced by the nature of the sample, since certain sample solvents promote better analyte retention on one sorbent group than another. For example, an aqueous sample high in salt containing an analyte with non-polar functional groups (e.g. a steroid in urine) should be screened on non-polar sorbents. An aqueous sample of low ionic strength containing an acid-bearing analyte (e.g. organic acids in wine) should be screened on anion-exchange sorbents. Conversely, an organic solvent containing analytes with hydroxyl or amine groups (e.g. a chloroform extract containing polyamines) might best be screened on polar sorbents. For some samples more than one choice of sorbent group may be possible.

To assist users in the choice of ISOLUTE SPE column or sorbent when developing a new sample preparation method, IST has developed an 11 - page '*Easy Step by Step Guide to Method Development*'. This guide takes you through the decision making process in SPE method development, covering the following aspects:

- *Determine sample size*
- *Select a retention mechanism*
- *Select a sorbent*
- *Determine sample pre-treatment*
- *Optimise column conditioning*
- *Determine sample loading rates*
- *Select interference elution solvents*
- *Consider column drying step*
- *Select analyte elution solvents*



Sample pre-treatment

Sample pre-treatment enhances analyte retention.
Use the following chart to help determine what pre-treatment may be required:

AQUEOUS MATRIX

Type of Analyte	Type of Sorbent	Pre-treatment
Neutral or Neutralized	Hydrophobic (e.g., C2, C8, C18, ENV+)	Add 0.5 to 1 % organic solvent such as methanol to samples having volumes >100 mL. If analyte is ionizable, adjust sample pH to 2 units above pK_a for bases and 2 units below pK_a for acids.
Cation	Cation exchanger (CBA, SCX, C2, PRS)	Adjust pH with buffer to ensure charge on analyte (2 pH units below analyte pK_a). *IMPORTANT NOTES:* 1. If CBA phase is used, pH must not be below 7. For C2 phase, pH must not be below 5. 2. The ionic strength of the sample must not exceed 50mM for singly charged cation, or 100 mM for doubly charged cation. Samples with a high ionic strength (e.g. urine) must be diluted. 3. An appropriate buffer should be selected that will not compete with the analyte of interest. The following series lists ions on the left that will displace ions on their right: $Ba^{2+} > Ag^{+} > Ca^{2+} > Zn^{2+} > K^{+} > NH^{4+} > H^{+} > Li^{+}$
Anion	Anion exchanger (NH2, SAX, PSA)	Adjust pH with buffer to ensure charge on analyte (2 pH units above analyte pK_a). *IMPORTANT NOTES:* 1. If NH2 phase is used, sample pH must not be above 7.8. 2. The ionic strength of the sample must not exceed 50mM for singly charged anion, or 100 mM for doubly charged anion. Samples with a high ionic strength (e.g. urine) must be diluted. 3. An appropriate buffer should be selected that will not compete with the analyte of interest. The following series lists ions on the right that will displace ions on their left: OH^{-} > acetate> formate> $HPO_4^{2-} > HCO_3^{-} > Cl^{-} > HSO_3^{-}$ > Citrate

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Part of IST's 11-page 'Step by Step Guide to Method Development' includes assistance with sample pre-treatment

Additional assistance with method development is available. Contact your IST distributor with your method development requests (see pages 5-7). Information on availability of IST's Technical Notes on the use of specific ISOLUTE SPE columns can be found in the SPE column ordering information pages of the SPE columns of interest (pages 18-49).

For rapid delivery of up to date technical notes, please register at the IST website

INTERNATIONAL SORBENT TECHNOLOGY

A guide to solid phase extraction

SPE Modes of Operation

There are two typical ways of using SPE and the choice of SPE mode depends on many factors. The need for trace enrichment, minimal or maximum purification or whether a diverse range of analytes must be simultaneously extracted, will all influence the approach to be taken. The nature of the matrix, the characteristics of analytes and interferences will also influence the ultimate choice.

SPE is most commonly used in applications that require simultaneous trace enrichment and purification of the sample. This mode involves the following six steps:

1. *Sample pre-treatment*
2. *Column conditioning*
3. *Column equilibration*
4. *Sample application*
5. *Interference elution*
6. *Analyte elution*

These same elements apply equally to extractions using the following retention mechanisms:

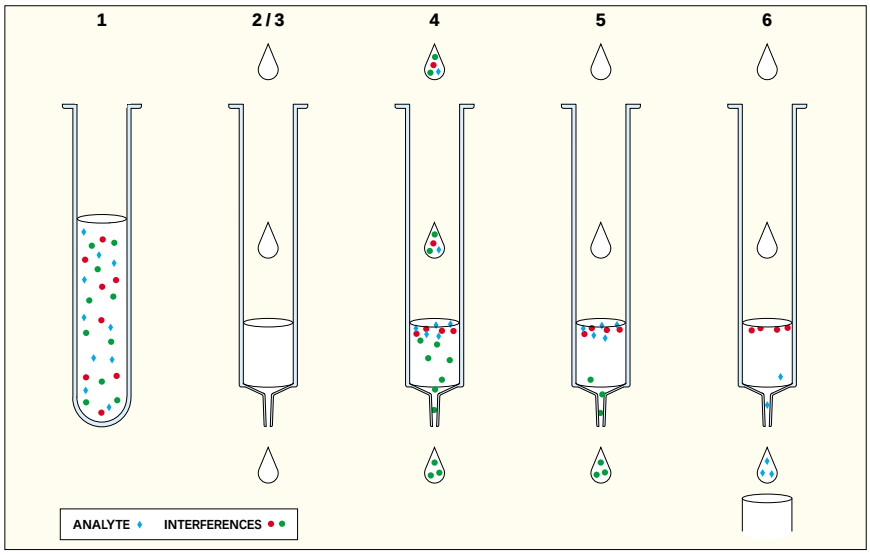
- *non-polar*
- *ion exchange*
- *mixed-mode*

Special cases such as the use of layered or stacked column arrangements also fall into this category.

Mixed-mode and layered column arrangements will be discussed separately in this guide, as they include special features that provide solutions to specific sample preparation situations.

The second most common mode of operation in SPE is used when **purification** only is required. This mode has four important elements, that generally apply to polar retention mechanisms :

1. *Sample pre-treatment*
2. *Column conditioning*
3. *Column equilibration*
4. *Sample application and collection*



1. SPE for simultaneous trace enrichment and purification

1.1 Non-polar and ion exchange retention mechanisms

Sample pre-treatment:

This step involves preparing the sample both physically and chemically for the SPE extraction in order to optimise conditions for the chosen extraction mechanism. The type of sample pre-treatment will differ, depending on the stability of the analytes, type of matrix, the nature of the interactions between the analytes with the sorbent and type of sorbent.

Aqueous samples: Reagents may be added to stabilise the analyte. For aqueous samples containing analytes that are to be retained primarily by hydrophobic interactions, pH adjustment may be required to ensure that the surface and analyte (if ionisable) are not charged. It may be necessary to add 0.5 to 1% wetting agent (e.g. methanol) to large volume samples (> 100 ml) to maintain an active sorbent surface. Where the primary interaction for analyte retention is ion exchange, the pH should be controlled to ensure that total ionisation of the analyte and surface of the sorbent has occurred.

Ionic strength must also be controlled in order to facilitate maximum retention of analytes. The ionic strength of the sample should be reduced to < 0.05 M by dilution with deionised water or low ionic strength buffer. The selectivity of the buffer cation (for cation exchange) or anion (for anion exchange) should be considered. Buffers that contain ions of lower selectivity than the analyte itself facilitate analyte retention. For cation exchange sorbents, the selectivity of the counter ion is affected by the degree of hydrophobic character of the sorbent. In cation exchange sorbents exhibiting minimal hydrophobic character (e.g. ISOLUTE SCX-2, a propylsulphonic acid phase), there is no significant difference in the selectivity of the counter ion, whereas, for more hydrophobic cation exchange sorbents (such as ISOLUTE SCX-3, an ethylbenzene sulphonic acid phase), the effect of selectivity is more pronounced.

Selectivity of some common anions (ions on the right will displace those on the left): OH^- < acetate < formate < HCO_3^- < Cl^- < HSO_3^- < CN^- < citrate < benzene sulphonate

Aqueous and non-aqueous samples: Dilution may be necessary to reduce sample viscosity, to ensure a free-flowing sample.

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SPE column conditioning:

Conditioning is usually necessary to prepare the SPE column for the extraction process. For retention of analytes to occur, the bonded phase must be able to interact with the sample matrix (see figure 5). A solvent is passed through the column to 'wet' the sorbent, and ensure interaction. The sorbent bed should not be allowed to dry out after solvation.
N.B. Column conditioning is not required for certain modified resins, e.g. ISOLUTE ENV+ (see figure 6).

Aqueous samples: The sorbent is wetted with an organic solvent such as methanol.

Non-aqueous samples: The sorbent is wetted with the matrix solvent.

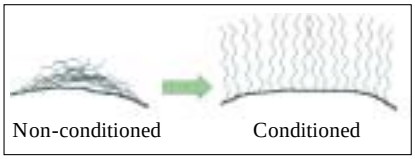


Fig. 5 Effect of conditioning on C18 bonded silica

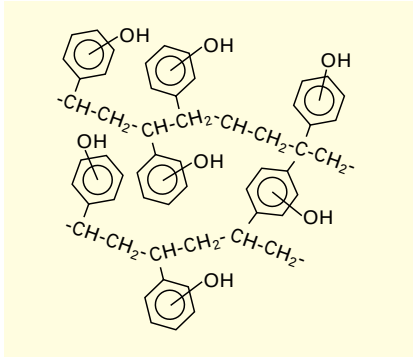


Fig. 6 The hydroxylated surface of ENV+ negates the need for conditioning

SPE column equilibration:

To maximise retention of the analyte by the sorbent, the column is treated with a solvent that is as 'matrix-like' as possible. A typical volume of equilibration solvent is 0.5-2 ml / 100 mg of sorbent. The sorbent bed should not be allowed to dry out between equilibration and sample loading.

Aqueous samples: The equilibration solvent should be similar to the sample matrix with respect to pH and ionic strength. If ion exchange is being used as the analyte retention mechanism, the ionic strength should be < 0.05 M. This step is often used to ensure the presence of an appropriate counter ion on an ion exchange column. See sample pre-treatment for counter ion selection.

Non-aqueous samples: Not required, since the previous step is typically performed using the matrix solvent.

Sample loading:

Optimisation of loading flow rates is an important part of method development. A good starting point is 1 ml / minute for a 1 ml cartridge, 3 ml / minute for a 3 ml column and 7 ml / minute for a 6 ml column (wider diameter columns yield lower linear velocities). The flow rate can be increased after the method chemistry is optimised. Flow rate is increased until some sample breakthrough is seen (as indicated by a drop in recovery). A flow rate slightly lower than the upper limit should be used. The optimum flow rate should be controlled and recorded to ensure reproducibility.

Interference elution:

The purpose of interference elution is to selectively remove undesired compounds from the sorbent without eluting the analytes. Ideally, a solvent is selected which is miscible with the sample matrix and in which the analytes are poorly soluble. A typical volume of interference elution solvent is 1-2 ml / 100 mg of sorbent. The flow rate should be adjusted such that the solvent is in contact with the sorbent for 1-2 min.

Aqueous samples: Ionic strength and pH control should be maintained at this stage to prevent analyte loss. A good choice of solvent is often the equilibration buffer. A buffer containing 10-30 % methanol or acetonitrile is often suitable for removing lipophilic interferences. Column drying may be necessary to remove water if the elution solvent is water immiscible. Drying can be performed by vacuum aspiration, N_2 or CO_2 flow, or centrifugation (useful if the analytes are volatile). Drying times depend on factors such as sorbent type and mass, bed dimensions, solvent to be selected for elution, and drying method. Depending on these factors and the degree of dryness required, drying times can range from 30 seconds to 30 minutes. If a water miscible elution solvent is selected, column drying can be reduced or eliminated. If the drying step is reduced, traces of water may be eluted in the elution step. Care should be taken to avoid phase separation or analyte precipitation on subsequent evaporation.

See section 1.2.1, 'Mixed-mode retention mechanism', for details on the special interference elution wash steps that can be used with mixed-mode sorbents to achieve extremely high purity extracts.

Elution:

The elution solvent should be one in which the analytes are soluble. It must often overcome primary and secondary retention mechanisms, and so a solvent or mixture of solvents offering multiple interactions is usually most effective. The elution solvent should be compatible with the final analysis technique. For example, for HPLC analysis, a solvent similar to the mobile phase is a good choice of elution solvent. A volatile solvent is generally selected for subsequent GC analysis. Other factors to consider include whether there will be a derivatisation step, as well as volatility of the solvent if further concentration is required. A minimum volume of elution solvent allows maximum concentration of the analytes. A typical minimum elution volume is 250 µl / 100 mg of sorbent. Flow control is important to ensure reproducibility. The use of two small aliquots of solvent with a 1 - 4 minute soak step between elution volumes is often more efficient than one large aliquot. If a single elution is required, the flow rate of the elution solvent should be such that contact time between solvent and sorbent is 1-4 minutes.

Aqueous samples: A water miscible elution solvent may be used to elute analytes and minimise or eliminate column drying (see interference elution in previous step). For analytes that are retained by ion exchange, high ionic strength (> 0.1 M) buffers can be used for elution. The high concentration of ions in the buffer will compete with an ionic analyte for ion exchange sites on the sorbent, thus causing elution of the analyte. For doubly charged analytes, buffers of > 0.2 M should be used. Buffers containing ions with a higher affinity for the sorbent than the analyte can be used for elution by displacement of the ionic analyte (see selectivities in sample pre-treatment section). An organic component in the elution solvent may be necessary to overcome secondary hydrophobic interactions.

Summary:

SPE combining both trace enrichment and purification is the most common approach in solid phase sample preparation. In each of the steps, conditions must be optimised for interactions between the analyte, matrix and sorbent. These conditions include pH, ionic strength, solvent strength, solvent volumes and flow rates.

1.2 Mixed-mode retention mechanism

1.2.1 Case I - Maximum extract purity

The mixed-mode approach to the extraction of ionisable compounds from aqueous biological matrices is a powerful way to achieve highly purified extracts.

Many drugs with a generally non-polar structure also contain an ionic group such as a primary or secondary amine, or an acid. There are therefore two possible retention mechanisms for extraction of these analytes.

The extraction of drugs from biological fluids using a purely non-polar retention mechanism can lead to extracts that contain a large amount of non-polar co-extracted material, which can interfere with the subsequent analysis. Conversely extraction mechanisms based on ion exchange interactions alone can be non-robust due to the variable ionic strength of the sample matrix.

The mixed-mode approach utilises special phases that combine a non-polar retention mechanism with an ion exchange (either cationic or anionic) retention mechanism. Once the column has been conditioned and equilibrated, the sample is loaded and analytes are initially retained through a non-polar retention mechanism, unaffected by the ionic strength of the sample. A rigorous elution regime can then be used to elute interferences retained by either ionic or non-polar interactions alone. Only analytes exhibiting both non-polar and ionic characteristics are retained on the column, subsequently to be eluted as an extremely pure extract.

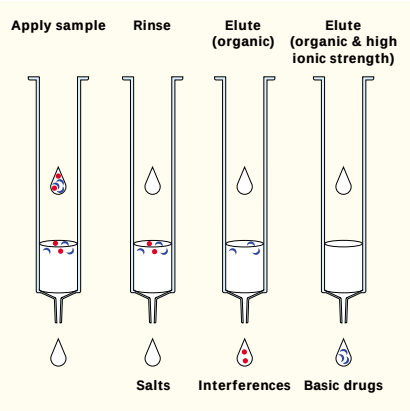
Sample pre-treatment:

This should be as for the main method, with the matrix optimised for the efficient retention of the analyte based on the non-polar retention mechanism.

Column conditioning, column equilibration and sample loading steps should all be carried out as described in the main method.

Interference elution:

The utility of mixed-mode columns is realised during the interference elution step. If the analyte is retained initially by hydrophobic interactions, then an initial rinse with an aqueous solvent of low ionic strength can be used to displace interfering ionic species. This can be followed with a rinse by an organic solvent to remove lipophilic interferences. Appropriate pH conditions should be maintained so that the analyte is charged during the organic rinse step, and transfer of the analyte to ion exchange sites is ensured.



Analyte elution

This should be carried out as described in the main method, with a solvent capable of breaking both non-polar and ionic retention mechanisms. For basic analytes, the elution solvent would normally involve using a base and appropriate organic solvent. This eliminates the positive charge on the analyte and overcomes the non-polar interactions respectively.

1.2.2 Case II: Fractionation of acidic, basic and / or neutral analytes

Sample pre-treatment:

The matrix is optimised for the efficient retention of the analytes based on both ion exchange and hydrophobic interactions. For example, the extraction of acidic, basic and neutral compounds can be performed on HCX (hydrophobic phase / strong cation exchange). In this case, the pH is adjusted to neutralise the acidic compounds, and ionise the basic compounds. The acidic and neutral compounds will be retained by hydrophobic interactions, while the basic compounds will be retained primarily by ion exchange.

Column conditioning:

As described in the main method.

Column equilibration:

As described in the main method, and consistent with mechanisms selected for sample pre-treatment.

Sample loading and interference elution:

As described in the main method.

Analyte elution:

Analytes can be selectively eluted by judicious choice of elution solvent. For the example given above, the acidic and neutral analytes can be eluted with an organic solvent. Conditions can be maintained such that the basic compounds continue to be retained during this first elution. Basic compounds can then be eluted with a suitable solvent.

Summary:

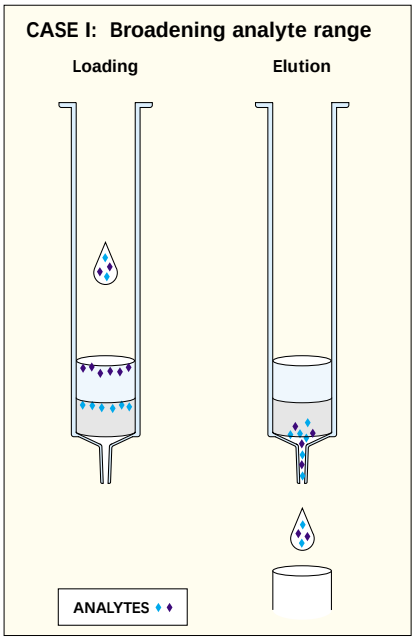
Mixed-mode columns are available for applications for both acidic and basic compounds. They are useful for producing very clean extracts, as well as for fractionating mixtures of compounds.

1.3 Layered columns

Layered columns are used to extract species that can be retained by different types of interactions, or degree of interaction. Two sorbents offering different interactions are contained in the same column. Layered columns can be used in either of two ways.

CASE I: Extending the analyte range

Samples containing analytes with a broad range of properties, where different phases would be appropriate to optimise retention as well as elution, can be extracted using layered phases. For example, a sample may contain both non-polar and polar analytes. A hydrophobic phase modified with a short chain hydrocarbon (e.g. C2) would be useful for retaining the non-polar compounds, but would be inadequate for retaining the polar species. Conversely, a hydrophobic phase such as C18 would retain both polar and non-polar species, but elution of the latter from a C18 phase is often difficult. Application of a layered C2 / C18 column accommodates the retention and elution of the full range of analytes. This format is easily automatable and is particularly suitable for many environmental applications (page 90).



Sample pre-treatment, column conditioning, equilibration, sample loading, and interference elution:

These steps should be carried out as described in the main method.

Analyte elution:

Analytes should be eluted as described in the main method. Elution conditions should be such that the elution solvent is strong enough to solvate analytes eluted from the top phase, so that they pass unretained through the bottom layer.

CASE II: Interference removal

Samples containing analytes and interferences with differing properties can be retained on different layered phases. The interferences are retained on the top layer, while the analytes are retained on the bottom layer. Elution conditions are selected such that the interferences continue to be retained while the analytes are eluted. This method utilising layered phases is amenable to automation. The ISOLUTE PAH column (page 90) is a good example of this approach.

Sample pre-treatment:

This should be as for the main method, with the matrix optimised for the efficient retention of interferences on the top phase and analytes of interest on the bottom phase.

Column conditioning, column equilibration and sample loading:

These steps should be carried out as described in the main method.

Interference elution:

Elute interferences that were not retained on the top layer as described in the main method.

Analyte elution:

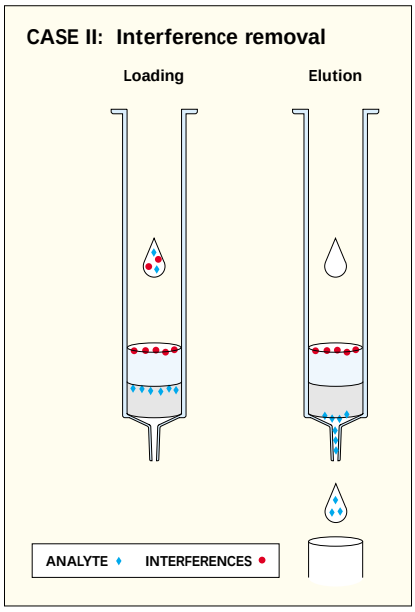
As described in the main method. Elution conditions should be such that interferences are retained on top layer, while analytes are eluted from bottom layer.

Summary:

The layered column mode allows for an improvement in selectivity when interferences can be retained by a different mechanism to the analytes. It also broadens the analyte range when analytes differ in the degree to which they are retained on similar types of sorbents. As the sorbents offering different retention mechanisms are contained within the same SPE column, both of these approaches are easily automatable.

Stacked columns

Both of the approaches described for layered columns can be performed using a stacked column arrangement. Sorbents offering different mechanisms are packed into separate SPE columns, and the columns are stacked vertically using an ISOLUTE column adaptor. This arrangement is ideal for method development and optimisation, but is not amenable to automation.



2. SPE for sample purification

SPE for purification only is commonly used when trace enrichment is not required, but interferences must be removed. The most common application is the removal of polar interferences from a non-polar solvent extract using a polar SPE column such as ISOLUTE NH2 or ISOLUTE SI. The interferences are retained on the column, while the analytes of interest pass through the sorbent bed and are collected with the sample solvent. When sorbent selection and sample solvent conditions are optimised, a highly purified extract can often be produced. This mode is easily automated. The steps involved are:

1. Sample pre-treatment
2. Column conditioning
3. Column equilibration
4. Sample loading and collection

Sample pre-treatment:

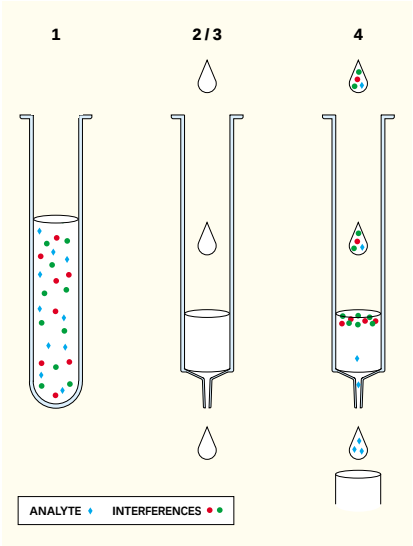
As for the main concentration and purification method, except that the conditions are selected in order to optimise retention of interferences, while minimising interactions between the sorbent and analytes.

As an example, for a non-polar analyte, to remove polar interferences using a polar sorbent phase, the sample environment should be made as non-polar as possible by dilution with a non-polar solvent ('matrix solvent').

Column conditioning and column equilibration:

As for main method, with conditions being chosen to maximise retention of interferences and minimise retention of analytes. Maximum retention of polar interferences on a polar column is aided by conditioning with a non-polar solvent.

In most cases, these two steps can be performed in one operation, as the sorbent is conditioned with the 'matrix solvent'.



Sample loading:

Sample should be loaded as described in the main method. Increase loading rate until breakthrough of interfering species is observed.

THE ELUENT CONTAINS THE ANALYTES AND SHOULD BE COLLECTED. FURTHER PROCESSING MAY BE REQUIRED.

Summary:

The four-step mode should be selected when interferences are present, but analyte concentration is not required. It differs from the six-step procedure primarily in that the sample loading step is also the step in which the analytes are eluted.

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INVITED ARTICLE

Assessing the effects of adsorptive polymeric resin additions on fungal secondary metabolite chemical diversity

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Adsorptive polymeric resins have been occasionally described to enhance the production of specific secondary metabolites (SMs) of interest. Methods that induce the expression of new chemical entities in fungal fermentations may lead to the discovery of new bioactive molecules and should be addressed as possible tools for the creation of new microbial chemical libraries for drug lead discovery. Herein, we apply both biological activity and chemical evaluations to assess the use of adsorptive resins as tools for the differential expression of SMs in fungal strain sets. Data automation approaches were applied to ultra high performance liquid chromatography analysis of extracts to evaluate the general influence in generating new chemical entities or in changing the production of specific SMs by fungi grown in the presence of resins and different base media.

Keywords: HPLC-Studio; fungal fermentations; Diaion® resin; Amberlite® resin; secondary metabolites; chemical diversity

1. Introduction

Fungal metabolites have historically provided a rich source of lead compounds for drug discovery. However, a general perception is emerging that rates of discovery of new molecules, especially new antibiotics, are decreasing after half a century of continued screening of fungal diversity by fungal fermentation processes (Bills et al. 2009). Recent advances in genome sequencing, facilitated by lowered costs and fast service, have revealed untapped reservoirs of microbial natural products in unexpressed biosynthetic pathways. The number of presumable secondary metabolite (SM) gene clusters in fungal genome analysed so far widely exceeds the number of detected metabolites for these fungal strains (Bergmann et al. 2007; Gross 2007; Hornung et al. 2007). This highlights the opportunity of finding possibly unexpressed genes that potentially could encode the biosynthesis of novel SMs, yet unknown because of our inability to simulate *in vitro* their environmental conditions required for expression (Gross 2007).

The value of empirical strategies for manipulating nutritional and environmental parameters to stimulate the metabolic diversity of a microorganism has long been recognized. In many industrial microbial screening laboratories, empirical comparisons of an organism metabolic response to a large number of medium formulations, over a range of temperatures and aerations, were often the first-line approach for improving product formation and associated bioactivity from a newly discovered fungus or actinomycete. The impact of varied media formulations was also evident during the initial fermentation of large numbers of new strains and testing extracts across multiple biological assays (Yarbrough et al. 1993). Review of screening data often recognized that interesting active metabolites were formed or produced in larger quantities in only one of several media. Recently, several approaches have been applied to foster the expression of these unexpressed pathways and to promote SM biosynthesis. These strategies include the manipulation of medium and growth conditions in miniaturized nutritional arrays (Bills et al. 2008), the application of transformation techniques for the generation of gene knockouts, the exchange of native gene promoters with constitutive or inducible promoters or the overexpression of transcription factors (Brakhage and Schroeckh 2011), the co-cultivation of one or more microorganisms in constant interaction (Combès et al. 2012), the addition of small-molecule elicitors such as epigenetic modifiers, inhibitors of histone deacetylase or DNA methyltransferase and the fermentation in the presence of non-ionic adsorption resins (de la Cruz et al. 2012).

Adsorptive polymeric resins have been incorporated since 1970s into fermentation processes for myxobacteria,

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fungi and actinomycetes, basically in order to increase titres by sequestering metabolites, preventing degradation or decreasing the cytotoxic effects of the final metabolites produced (Phillips et al. 2013). Several examples of microbial SMs demonstrate that the addition of resins cause some of these effects, including: (a) the production of rubradirin, an antibiotic produced by *Streptomyces achromogenes* subsp. *rubradiris* (Marshall et al. 1990) in which the addition of non-ionic polymeric adsorbents XAD-2, XAD-7, XAD-16, HP-20 and HP-21 led to a two- to fourfold increase in rubradirin titres after 3–4 days of fermentation; (b) paulomycin production, wherein the resin prevented the transformation of paulomycin to paulomenol (Marshall et al. 1987); (c) the production of BMS-182123, a metabolite produced by *Penicillium chrysogenum* which acts as an inhibitor of tumour necrosis factor alpha (TNF-α), whose production was increased by 5.5-fold by adding XAD-8 resin in the fermentation medium (Warr et al. 1996); (d) the production of migrastatin and isomigrastatin, potential anticancer agents produced by *Streptomyces platensis* (Woo et al. 2002), where XAD-16 added to the fermentation medium enhanced its production; (e) the production of ambruticins and jerangolids, new antifungal compounds produced by the myxobacterium *Sorangium cellulosum* (Gerth et al. 1996) and (f) the production of nomimicin, a new spirotetronate class of polyketide obtained from an actinomycete of the *Actinomadura* genus fermented in the presence of the polymeric resin HP-20 (Igarashi et al. 2012).

Our research group also described recently that the production of some metabolites is increased in the presence of non-ionic adsorption resins such as Amberlite® XAD-7 (de la Cruz et al. 2012), sometimes resulting in a modulation of whole-cell antibiotic production. So far, no clear evidence indicated that the addition of non-ionic adsorptive resins to fermentation processes is capable of activating unexpressed biosynthetic pathways, or increasing the chemical diversity produced in fungal fermentations (Phillips et al. 2013). To examine this hypothesis, we have evaluated the effect of different resin additions on fungal SM diversity production using ultra high performance liquid chromatography (uHPLC)-UV profiles and a new data-automated analysis method. Our aim was to generate collections of natural product extracts with high chemical diversity, with the final goal of finding new potential non-cytotoxic antifungal and antibacterial agents in our drug discovery programs.

2. Material and methods

2.1. Inocula preparation

Cryotubes containing fungal inocula or mycelia discs in 10% (v/v) glycerol stored at −80°C were thawed, and strains were revived and transferred to 60-mm Petri dishes containing 10 ml of YM agar (malt extract 10 g, yeast extract 2 g, agar 20 g, 1000 ml distilled H_2O) at 22°C for 14–20 days. Five mycelial discs were cut from each 60-mm plate with a sterile Transfer Tube (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA). Mycelia discs were extruded from the Transfer Tube and crushed in the bottom of tubes (25 × 150 mm) containing 12 ml of SMYA medium (Difco™ neopeptone 10 g, maltose 40 g, Difco™ yeast extract 10 g, agar 4 g, distilled H_2O 1000 ml) and two cover glasses (22 × 22 mm). Tubes were incubated on an orbital shaker (200 rpm; 1.5 cm throw), where rotation of the cover glasses continually sheared hyphae and mycelial disc fragments to produce nearly homogenous hyphal suspensions consisting of minute hyphal aggregates and fine mycelia pellets. Tubes were incubated for 7 days at 22°C (Bills et al. 2008).

2.2. Media formulation

MMK2 (mannitol 40 g, Difco™ yeast extract 1 g, Sigma-Aldrich (St. Louis, MO, USA) Murashige & Skoog salt 4.3 g, distilled H_2O 1000 ml), SCAS (Panreac (Barcelona, Spain) soluble starch from potato 40 g, Sigma-Aldrich casein hydrolysate 5 g, KH_2PO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.01 g and distilled H_2O 1000 ml), MV8 (maltose 75 g, V8 juice 200 ml, Sigma-Aldrich soy flour 1 g, L-proline 3 g, 2-(N-morpholino)ethanesulfonic acid 16.2 g and distilled H_2O 800 ml), YES (Difco™ yeast extract 20 g, sucrose 150 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, trace elements 1 ml ($ZnSO_4 \cdot 7H_2O$ 1 g/100 ml and $CuSO_4 \cdot 5H_2O$ 0.5 g/100 ml) and distilled H_2O 1000 ml), LSFM (glycerol 18.7 g, glucose 40 g, NH_4SO_4 2 g, Sigma-Aldrich yeast autolysate 5 g, soybean flour 5 g, tomato paste 5 g, sodium citrate 2 g and distilled H_2O 1000 ml) and XPMK (D-Xylose 60 g, Difco™ peptone 8 g, KH_2PO_4 0.5 g, Murashige & Skoog salt 4.3 g, distilled H_2O 1000 ml). Fermentation vials (42 ml EPA vials) containing 10 ml of medium were autoclaved for 21 min at 121°C.

2.3. Addition of adsorptive resins

Four adsorptive polymeric resins were added to fermentations: Amberlite® XAD-2, XAD-7, XAD-16N (hydrophobic polyaromatic resins, Sigma-Aldrich) and Diaion® HP-20 (styrene-divinylbenzene, Supelco (Bellefonte, PA, USA)) polyaromatic adsorbent resin for hydrophobic retention of compounds as antibiotics and other molecules. The resins were washed in twice their volume of methanol and stirred for 1 h, followed by six washes with distilled water, and were finally stored at 4°C in distilled water for 48 h (Singh et al. 2010). Afterwards, they were vacuum filtered through a 125 mm diameter and 6 µm pore size filter. Once filtered, the resins were oven dried at 75°C, and 0.3 g of each resin was added to the fermentation vials before autoclaving for 21 min at 121°C.

2.4. Growth

Cultures of each fungus were inoculated by adding 0.3 ml of inoculum suspension into five 42-ml EPA scintillation vials containing 10 ml of culture media with and without each of the resins. The vials were incubated for 14 days in a shaking incubator (Kühner AG, Birsfelden, Suiza) at 22°C, 220 rpm and 70% relative humidity.

2.5. Extraction

After 14 days of incubation, fermentation broths with cells were extracted by adding 9 ml of acetone using a Multiprobe II robotic liquid handler and shaking at 220 rpm for 1 h. After centrifugation, 12 ml of supernatant from each vial was transferred to glass tubes containing 0.6 ml of dimethyl sulfoxide (DMSO). Solvent was evaporated under a hot nitrogen stream to a final volume of 3 ml (80/20 water/DMSO solution) and a final concentration of 2× whole broth equivalents. Each fermentation batch included extracts from control culture media to discriminate their components. This extraction procedure was validated using antibiotics covering a wide range of polarities, molecular weights (MWs) and chemical structures, measuring in each case if compounds were detected after the extraction and preparation of the samples. For this purpose, water solutions containing 1 mg/ml of Terramycin®, cephalothin, amphotericin B, novobiocin, fusidic acid and actinomycin and unsaturated resins at 3% weight/volume were extracted applying the same methodology.

2.6. Bioactivity characterization

The antimicrobial activities of the extracts were evaluated against methicillin-resistant *Staphylococcus aureus* (MRSA) MB5393 (de la Cruz et al. 2012), *Candida albicans* MY1055 (de la Cruz et al. 2012) and *Aspergillus fumigatus* ATCC 46645 (Monteiro et al. 2012). Briefly, the microorganisms were incubated with the extracts for 18–30 h at 37°C. Sample 1:10 or 2:25 dilutions were used depending on the target strain. The activities were measured by monitoring the absorbance differences at 600 nm between the final and the initial incubation times, except for *A. fumigatus* where the activity was scored by using resazurin, an oxidation–reduction indicator of the cell viability (Monteiro et al. 2012). Additionally, the cytotoxicity of the different extracts against the HepG2 cell line (hepatocellular carcinoma, ATCC HB 8065) was evaluated by a classical 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction colorimetric assay, with the same incubation times and assay concentrations as used for the antibiotic evaluation (de Pedro et al. 2013).

2.7. Chemical analysis

Chemical profiles of fermentation extracts were analysed using an Agilent 1290 Infinity uHPLC-diode array detector (DAD). A Kinetex C-18 (1.7 μm, 2.1 × 150 mm) from Phenomenex (Torrance, CA, USA), a 10-min gradient from 1% to 99% (v/v) of acetonitrile in water, with 1.3 mM ammonium formate and 1.3 mM trifluoroacetic acid as chromatographic modifiers, a flow rate of 0.315 ml/min, a controlled temperature of 40°C and UV detection at 210, 280 and 340 nm were used for each analysis. An inert internal plastifier control (IC) was present in each sample to individually validate and normalize if necessary each chromatographic run. Additional methanol blanks were injected every 10 samples for joint monitoring of each analytical batch. Agilent integration uHPLC parameters included tangent skip mode (standard), tail peak skim height ration (0), front peal skim height ration (0), skim valley ratio (0), baseline correction (advanced), peak to valley ratio (1), slope sensitivity (15), peak width (0), area reject (0), height reject (0) and shoulders (TAN).

2.8. Data automation

The samples and positions in the storage plates were recorded in a computer database Oracle system (Nautilus LIMS by Thermo) where the producing microorganism, the fermentation medium, the additive, the extraction procedure and the location of each sample in the storage plates were recorded. An improved version of HPLC-Studio (Tormo et al. 2003; Tormo & García 2005) correlated all injected samples and extracts to their corresponding unfermented fermentation broths and allowed the chemical comparisons.

This recent version of HPLC-Studio (García & Tormo 2003; Tormo et al. 2003; Tormo & García 2005), with improvements for its application in uHPLC with Oracle databases (without limitations on the number of traces that could be processed at the same time), was used for data processing. No intensity peak thresholds were used. All the peaks eluting between 1.5 and 9.0 min were processed.

Variations found in the uHPLC retention times in the analysis of complex extracts by HPLC-DAD and uHPLC-DAD were due to several factors, such as the composition of the mixtures, the concentration of the compounds, the existence of molecules with very close retention times and the degree of resolution of the DAD detection and were corrected by applying a window of 0.065 min (double of the DAD scan detecting resolution), previously established as an adequate resolution time to distinguish between two distinct SMs among a large batch of sequential chromatograms (García & Tormo 2003; Tormo et al. 2003; Tormo & García 2005).

2.9. Characterization of chemical diversity by profile clustering

Only data related to the presence or absence of newly produced metabolites were used. Peak areas were not taken into account for the analysis to avoid biases due to very large

areas and to a possible deficient extraction of all quantities of the produced metabolites. Therefore, interpretations were strictly based on the presence or absence of peaks, without the quantitative data. To validate this approach, extracts of a water solution of several antibiotics (see Section 2.5) were also subjected to uHPLC analysis. Although in some cases the recovery titres were low (especially the one with the lowest polarity, actinomycin; see Supplementary information), the presence of all the antibiotics in the acetone extract confirmed that, in terms of presence or absence of the compounds, the extraction method could be used for chemical comparisons of the microbial SM profiles obtained under different fermentation conditions.

Components of the control fermentation media were subtracted from any SM counting. Chemical characterization of the diversity of the extracts started with the determination of the number of metabolites that were detected in the fermentations versus the control media. Next, the number of different peaks in a single condition versus the number of peaks of the metabolic footprint obtained from all the peaks present among all conditions for a single strain indicated the chemical diversity generated by that strain in each condition (a ratio expressed as a percentage). All fermentation conditions were classified based on their contribution to the chemical diversity following this procedure. The matrix of percentages of overlapping diversity between each condition was also determined.

BioNumerics software version 6.0 (Applied Maths) was used for the comparison of the metabolic footprints of each strain in the different fermentation conditions. The BioNumerics software was loaded with the resulting data matrix generated for the analysis of the common and uncommon peaks generated by the software (García & Tormo 2003; Tormo et al. 2003; Tormo & García 2005). The statistical analysis in BioNumerics was based on the determination of the Dice similarity coefficient (Dice 1945). From the estimated similarity coefficients, similarity dendrograms were generated by the unweighted pair group with arithmetic averages (UPGMA) method. This method uses the arithmetic average to generate clusters, showing the similarities between the different fermentation conditions based on the presence or absence of common SMs in each case. Principal component analysis (PCA) was also calculated with the BioNumerics software to facilitate visualization of relationships among the different fermentation conditions per strain.

2.10. *Chemical profiling validation for adsorptive resins comparisons*

Strategies for automated evaluation on SM profiles with automated HPLC peak database comparison have been outlined previously by our group to select the most chemically diverse condition before scale-up (García & Tormo 2003; Tormo et al. 2003; Tormo & García 2005). The same methods could be used statistically to assess the real influence on the metabolic profiles caused by the adsorptive polymeric resin additions. However, the degree of sensitivity and accuracy of the method had to be validated to confirm that the degree of variation observed within triplicates was always smaller than the one observed among the different fermentation conditions evaluated for the resins.

For this purpose, three different species of fungi were selected and fermented in triplicate, in two different base media each, with three different fermentation conditions (control media, control media plus XAD-16 and control media plus HP-20 additives). Chemical profile results proved that the previous method developed for the comparison of different metabolic profiles (presence or absence of detected metabolites by uHPLC at 210 nm and further statistical analysis by Dice + UPGMA from uHPLC-Studio + BioNumerics; Tormo & García 2005) was sufficiently sensitive that the triplicates had narrower distance coefficients than the statistical distances observed among the varied fermentation conditions.

3. Results and discussion

3.1. *Bioactivity evaluation of sets of strains*

Ninety-six different species of filamentous fungi from the Fundación MEDINA strains collection, belonging to orders known to be typical sources of biologically active molecules, were selected: *Chaetothyriales, Diaporthales, Dothideales, Eurotiales, Helotiales, Hypocreales* and *Pleosporales*. After 14 days of fermentation in MMK2 medium in the presence or absence of resins, differences in texture, colour, morphology and biomass were observed among the different resins when harvested (Figure 1).

Figure 1. Fungal strain CF-212941 grown on MMK2, MMK2 +HP-20, MMK2+XAD-2, MMK2+XAD-7 and MMK2+XAD-16 at 14 days, showing differences in texture, colour, morphology and quantity of biomass.

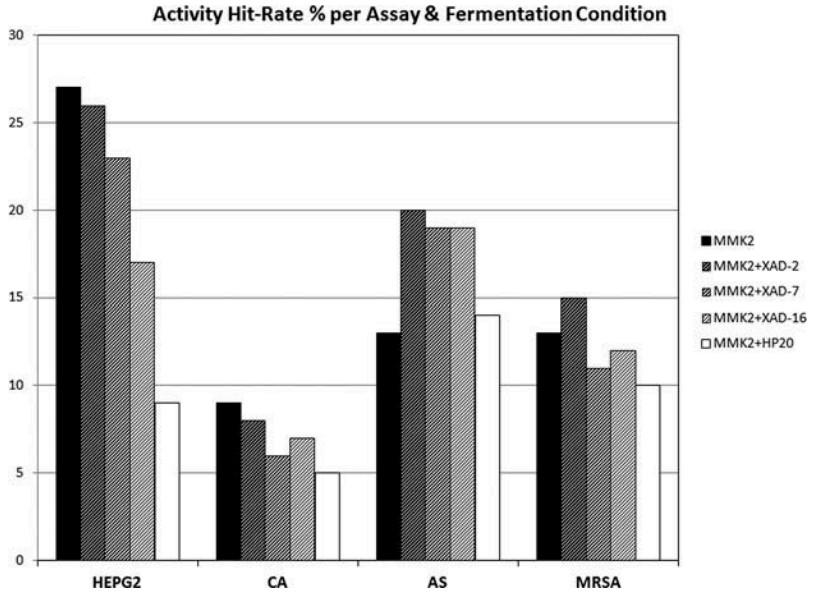

Figure 2. Activity hit-rate (%) of extracts, per assay and fermentation condition for the 96 fungal strains tested. HEPG2, cytotoxicity assay in HepG2 cell line. CA (*Candida albicans*), AS (*Aspergillus fumigatus*) and MRSA (methicillin-resistant *Staphylococcus aureus*) anti-infective tests.

Assuming that the phenotypic difference could indicate different SM production and therefore different potential new chemical entities, we proceeded to their extraction and evaluation for anti-infective and cytotoxic properties against three relevant human pathogens: one Gram-positive bacterium, MRSA and two fungi (*Candida albicans* and *Aspergillus fumigatus*). Since many antibacterial and antifungal metabolites also exhibit human cell cytotoxicity, this biological activity was also added to the assay panel (Figure 2).

Results indicated that, in general, for the MMK2 base fermentation medium, the addition of Diaion® HP-20 resin resulted in a decrease in cytotoxic and antimicrobial active hit-rates, whereas the addition of Amberlite® XAD-2 showed generally higher antimicrobial and cytotoxicity activity hit-rates than XAD-16 or XAD-7, which presented intermediate activity hit-rates.

Because most of the strains did not differ significantly in their activity profiles whether fermented in the presence or absence of the resins, we therefore focused further detailed studies on the subset of strains that exhibited consistent changes of their activity profiles when fermented in the presence of the resins, as a clear indicator of the existence of real changes in their metabolite profiles, including changes in metabolite concentrations. For this purpose, 14 strains from the original 96 were selected for chemical analysis because they presented consistent clear variation patterns on their activity profiles among the different target strains (Table 1). This subset of strains covered a wide taxonomic space for assessing the effect of the addition of the resins on a wide variety of fungi, including orders: Eurotiales (*Penicillium*), Hypocreales (*Cordyceps, Bionectria, Fusarium, Cosmospora*), Pleosporales (*Preussia, Phoma, Sydowiella*), Chaetothyriales and Diaporthales (*Pilidiella, Diaporthe*).

Among all the fermentation conditions tested, XAD-16 and HP-20 resins were also initially selected for further detailed studies because they were associated with larger differences in activity hit-rates versus base medium conditions (Figure 2). According to the manufacturers, both resins have similar chemical properties (styrene-divinylbenzene): XAD-16 is mostly used for adsorption of organic compounds of medium MWs from aqueous systems and polar solvents and HP-20 is used for refining of pharmaceuticals and natural extracts because it adsorbs larger molecules due to its relatively large pore sizes. XAD-16 characteristics included 560–710 µm of particle size, 200 Å of pore size and 800 m^2/g of surface area, whereas HP-20 characteristics included 250–850 µm of particle size, 260 Å of pore size and 500 m^2/g of surface area. The larger particle surface and slightly smaller pore size, but higher number of pores, make XAD-16 more suitable for hydrophobic compounds up to MW of 40,000, especially proteins. On the contrary, HP-20, with slightly larger pore sizes, can also be used for larger MW compounds.

3.2. *Effect of resins on the fungal growth*

Furthermore, in order to determine whether the changes in SM obtained by the fermentation in presence of the resin additives were due to changes in the initial composition of

Table 1. Antibiotic activities of the fungal strains selected.

Strain ID	TAXINIT	Country	HEPG2	CA	AS	MRSA
CF-194989	*Phoma* sp.	Puerto Rico	+++++	−+−−−	+++++	−+−−−
CF-195017	*Preussia* sp.	Puerto Rico	−+−−−	−−−−−	−+−−−	−+−−−
CF-209155	*Preussia intermedia*	Portugal	+−−−−	−−−−−	−−−−−	−−−++
CF-209171	*Preussia* sp.	South Africa	−+−+−	−+−+−	−+−+−	−+−+−
CF-209591	*Cordyceps* sp.	New Zealand	−++−−	−++++	−+++−	+++−−
CF-210345	*Sydowiella* sp.	Puerto Rico	−−−−−	−−−+−	−−−+−	−++++
CF-210367	*Cosmospora vilior*	Spain	+−−−−	−−−−−	+−−−−	+−−−−
CF-210370	*Bionectria* sp.	Spain	+−−−−	−−−−−	−−−−−	+−−−−
CF-210988	*Penicillium* sp.	C. African Republic	−−−+−	−−−−−	−++++	−−−+−
CF-210989	*Fusarium* sp.	C. African Republic	−−−+−	−−−−−	−−−−−	−+−−−
CF-214546	*Diaporthe* sp.	C. African Republic	−+−−−	−−−−−	−−−−−	−+−−+
CF-214552	*Pilidiella castaneicola*	New Zealand	+−−−−	+−−−−	−−−−−	+−−−−
CF-214558	*Penicillium* sp.	C. African Republic	−−−+−	−−−−−	−−−−−	−+−−−−
CF-214575	*Chaetothyriales* sp.	Spain	−−−−−	−++++	−++++	−−−−−

Notes: HEPG2: hepatocellular carcinoma ATCC HB 8065; CA: *Candida albicans* MY1055; AS: *Aspergillus fumigatus* ATCC 46645; MRSA: methicillin-resistant *Staphylococcus aureus* MB5393. Sequentially: MMK2 (±), MMK2+XAD2 (±), MMK2+XAD7 (±), MMK2+XAD16 (±) and MMK2+HP-20 (±).

the medium (i.e. by having the resin capturing essential components prior to the inoculation), we pre-treated the MMK2 control media with the resins prior to their filtration and autoclaving. The sucrose-rich medium (YES) was also tested to evaluate if possible differences could be medium dependent. YES medium was selected because of its historical success for enhancing the production of active SMs (Bills et al. 2008).

In general, the addition of the resins to the fermentations caused variations in the morphology, colour and amount of biomass in many of the broths, with fermentation triplicates behaving homogeneously. For example, in *Penicillium* sp. strain CF-210988 grown in YES medium (Figure 3), the treated and pre-treated conditions with XAD-16 or HP-20 produced more biomass than the control medium, with no substantial chemical differences between pre-treated and treated media. Interestingly, colour from either pre-treated or treated conditions was equally different when compared to the control condition, indicating that the pre-treatment with the resins had affected the growth to a certain extent. These effects might be explained by a potential sequestration by the resin of trace elements or some components of the medium during the pre-treatment.

However, in the case of the MMK2 medium, the XAD-16 pre-treated condition was very similar to the control medium, whereas the HP-20 pre-treated condition was more similar to both XAD-16- and HP-20-treated media. When the same strain was grown in MMK2 medium, only the XAD-16-treated medium exhibited increased biomass production and different chemical profile compared to the control. Interestingly, for both media, the HP-20 resin surface remained white after harvesting, whereas XAD-16 resin became strongly pigmented in both media (Figure 3).

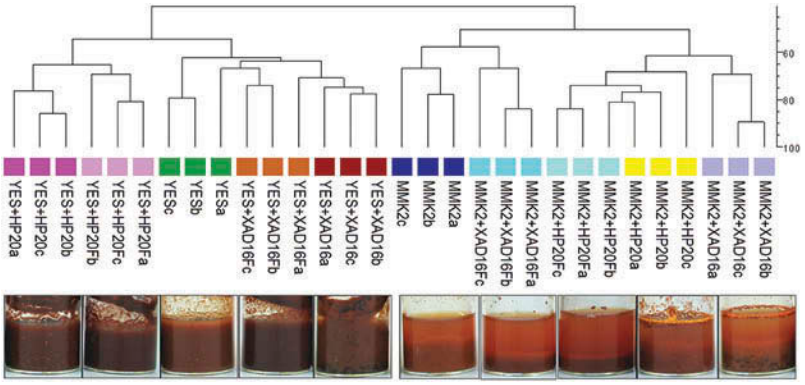

Figure 3. UPGMA dendrogram showing overall similarities between SMs produced by fungal strain (CF-210988) when grown on 10 different fermentation conditions per triplicate, comparing the different fermentation triplicates (a, b, c), the different resins (HP-20 or XAD-16) and two base media autoclaved in the presence of the resins or just pre-treated before inoculating the fungal strain (Fa, Fb, Fc). Similarities were determined by Dice's coefficient values.

In general, differential production profile and more biomass quantities were always observed when treatments with resins were performed along the whole fermentation processes. This observation and the literature references to the production of certain metabolites related to product displacement mechanisms along the whole fermentation process determined us to focus our further chemical profiling studies only on full-treated fermentation conditions.

3.3. Chemical evaluation of the resin addition to fungal fermentations

To characterize in detail the differences observed within the set of 14 fungal strains with or without adding the resins during the whole fermentation process, we decided to compare their SM profiles by uHPLC. Analysis of their SM chemical profiles (Figure 4) proved that for each of these strains, there could be: (i) conditions with clear improvement in the quantity of production of given compounds in the presence of the resins, (ii) compounds that only were produced in the control medium (without resins) and (iii) compounds that were only present when specific resins were added. The large number of cases found with variations in the presence or absence of specific SMs, determined by counting the variations in the number of compounds detected by uHPLC, led us to a practical representation on the real effect of the resins for this set of fungal strains.

Initially, the difference in the number of SMs produced by each strain with added resins was not significant for any of the four additives tested in the MMK2 medium (Figure 5). However, to determine the best condition for enhanced chemical diversity production, we scored the frequencies of the conditions that generated the maximum non-overlapping chemical diversity for each strain versus the total number of conditions used per strain. An averaged scoring was obtained for the representative subset of 14 strains (Table1, Figure 6). According to this methodology (García & Tormo 2003; Tormo et al. 2003; Tormo & García 2005), displacements of a condition from the 1:1 diagonal linear correlation clearly highlight different chemical diversity influences: (i) the greater the displacements towards the upper part of the graph, the better the medium results for exclusive new SM generation; whereas (ii) displacements towards the lower half of the graph indicate lesser differential metabolite generation (Figure 6).

This analysis clearly indicated that the order of influence of the resins in inducing more to less chemical diversity was sequentially XAD-16 followed by XAD-2, XAD-7 and HP-20. This result could be correlated, to a

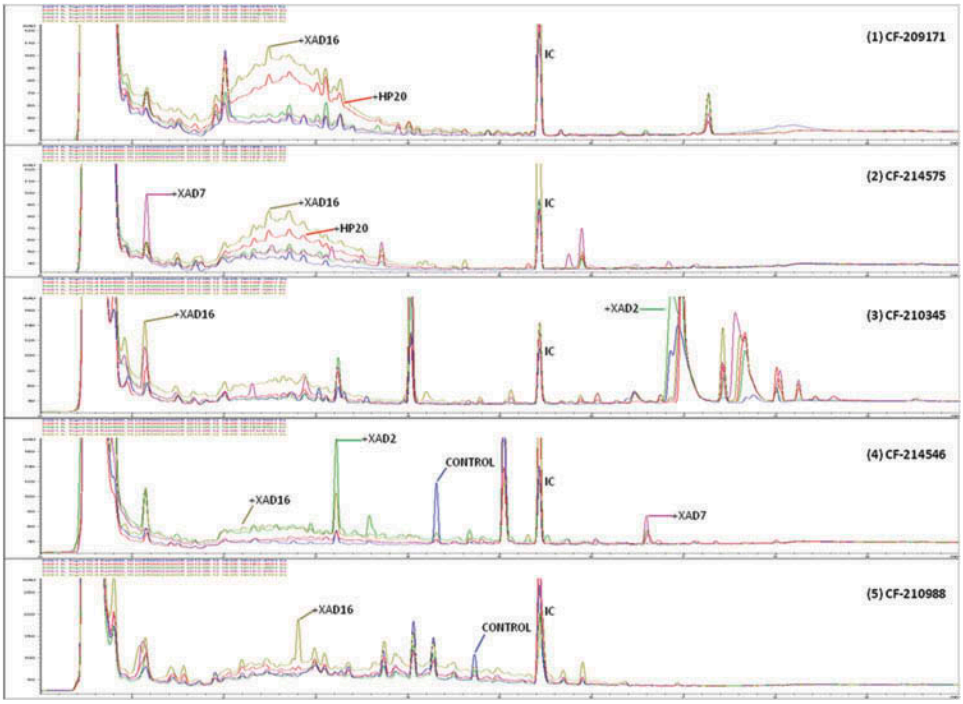

Figure 4. Comparative analysis of different uHPLC-UV 210 nm secondary metabolite profiles produced by five different fungal strains: (1) *Preussia* sp. (CF-209171); (2) *Chaetothyriales* sp. (CF-214575); (3) *Sydowiella* sp. (CF-210345); (4) *Diaporthe* sp. (CF-214546); (5) *Penicillium* sp. (CF-210988) when these fungus were grown on MMK2 fermentation medium with and without XAD-2, XAD-7, XAD-16 and HP-20 resins. Relevant uHPLC traces are indicated in the figure. Internal control (IC) was added homogeneously to each sample to allow accurate comparisons of the chromatographic runs.

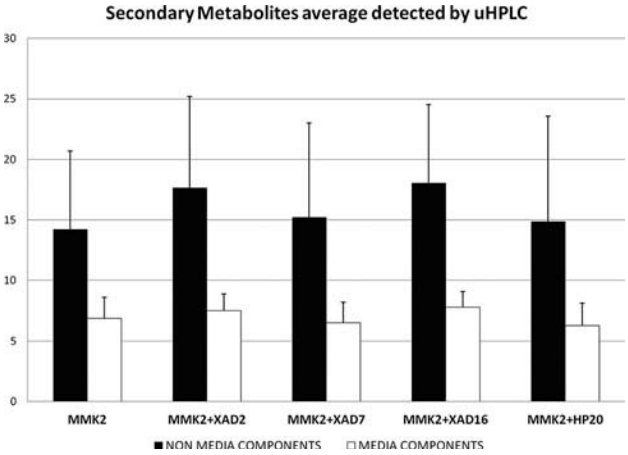

Figure 5. Average number of secondary metabolites detected by uHPLC-DAD for each additive to the MMK2 control medium fermentation for the 14 fungal strains that responded positively to the resin addition. The *T*-test method was used to compare pairs; pair differences were not statistically significant at $\alpha = 0.05$.

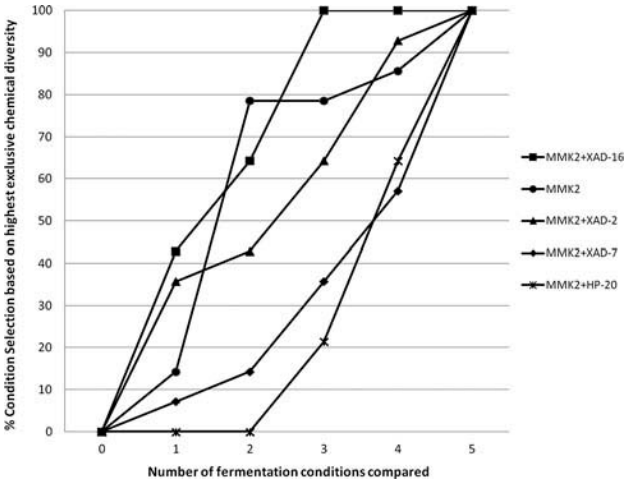

Figure 6. Percentage of fermentation conditions that presented highest exclusive chemical diversity as a function of number of fermentation conditions compared to evaluate the contribution to greater chemical diversity detected by uHPLC for five different fermentation conditions tested in 14 fungal strains that responded positively to the resin addition.

certain extent, with the low bioactivity hit-rates observed in the anti-infective and cytotoxic assays for the HP-20 resin (Figure 2), which in turn, could be related to the reduction in the chemical diversity also observed of these fermentations.

To determine whether these effects were dependent or not on the composition of the basal medium and, whether changes in carbon, nitrogen or trace elements sources could have greater influences on the production of SM than simply the presence of the resins, the same 14 fungi were also fermented in other five media with completely different compositions in carbon, nitrogen and trace elements sources (LSFM, MV8, SCAS, XPMK and YES) (Bills et al. 2008), with and without XAD-16 and HP-20 resins.

In general, and in agreement to the MMK2 results (Figure 5), the number of SMs produced did not differ significantly in any of the conditions (results not shown). Dice + UPGMA comparison of the chemical profiles for all 14 strains confirmed that the adsorptive polymeric resins generated slightly lower modifications in the SM profiles than changes in the composition of the base media (Figure 7A). This could also be observed by other statistical methods when principal components were calculated for this set of strains (Figure 7B). Clear grouping of all conditions belonging to each separate medium indicated that more chemical diversity was achieved by changing the medium composition than by adding the Diaion® or Amberlite® resins.

Specifically, the medium that generated a higher range of chemical diversity was LSFM followed by MV8, XPMK, YES and SCAS (Figure 8). This analysis also confirmed that changes in the composition of the fermentation medium determined higher variations in the chemical profiles than the addition of the resins. However, the detailed scoring of each condition (LSFM > LSFM+XAD-16 > LSFM+HP-20; MV8+XAD-16 > MV8 > MV8+HP-20, XPMK+HP-20 > XPMK > XPMK+XAD-16, YES +XAD-16 > YES+HP-20 > YES and SCAS+XAD-16 > SCAS+HP-20 > SCAS) indicated that, for fermentation media where a large chemical diversity production is initially observed, such as in LSFM, MV8 and MMK2, the fermentation media without resins generated similar or higher diversity than the one obtained by the addition of XAD-16 or HP-20. However, for fermentation media that induced lower metabolite diversity production, such as SCAS and YES, the addition of XAD-16 or HP-20 resulted in a clear improvement of the chemical diversity profiles generated (Figure 8).

3.4. *Unique secondary metabolites and changes in production obtained by resin addition*

During the statistical characterization of the chemical profiles generated with the addition of the resins, it became apparent that most of the fermentation conditions presented unique SMs and differential production titres of specific compounds. Figure 9 depicts an example (*Preussia* sp. CF-209171) where only MV8, SCAS and XMPK+PH-20 extracts resulted in inhibition zones against *Candia albicans*. In this strain, unique uHPLC peaks could be detected in 66% of the conditions: MV8, MV8+XAD-16, SCAS, SCAS+HP-20, SCAS+XAD-16, LSFM, LSFM+XAD-16, XPMK, XPMK+XAD-16 and XPMK+HP-20, and increments in the quantity of certain metabolites with respect to the control media could be

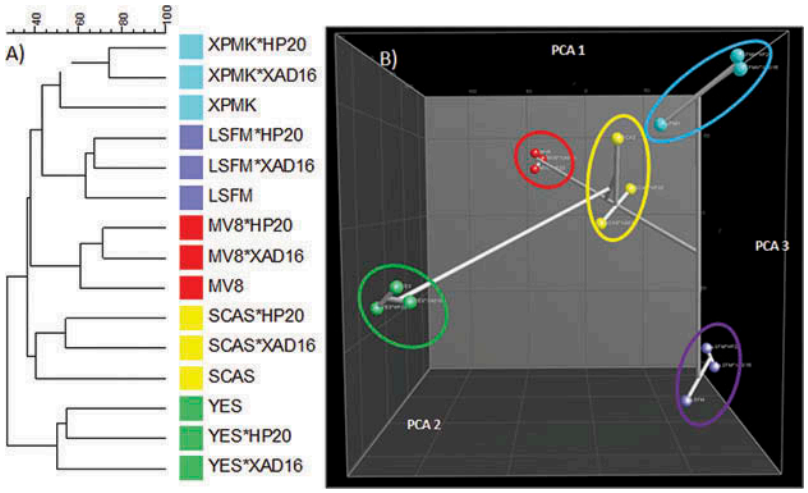

Figure 7. (A) DICE-UPGMA dendrogram. (B) Principal component analysis (PCA) of the secondary metabolites produced by the 14 selected strains from the study that responded positively to the resin addition grown on 15 fermentation conditions. PCA three-dimensional representation only depicts the three principal statistical components.

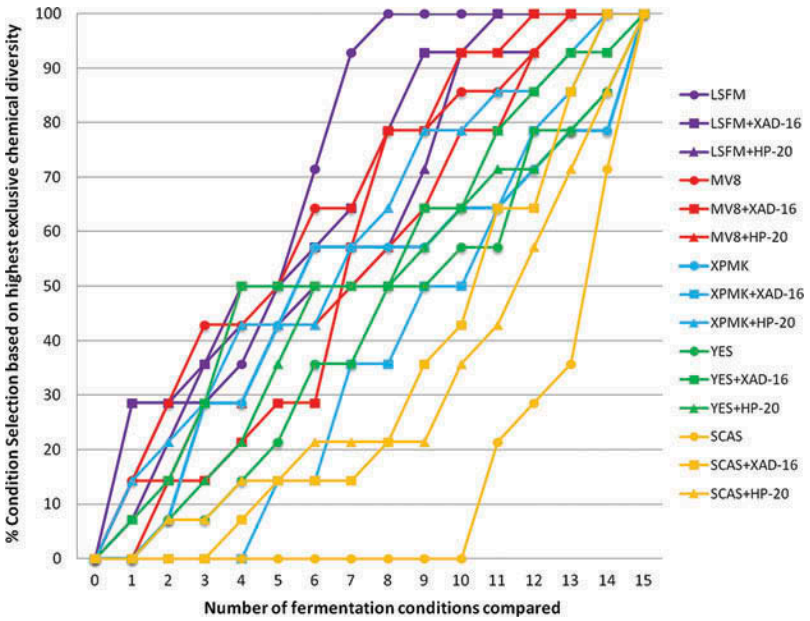

Figure 8. Ranking in percentage of the fermentation conditions that presented the highest exclusive chemical diversity as a function of number of all fermentation conditions compared per strain. To evaluate the contribution to greater chemical diversity detected by uHPLC, 15 different fermentation conditions were tested for the 14 selected fungal strains that responded positively to the resin addition.

detected in 50% of the resin conditions: MV8+XAD-16, SCAS+XAD-16, LSFM+XAD-16, XMPK+XAD-16 and YES+XAD-16.

Scoring on the number of unique metabolites per condition in the set of 14 strains supported this hypothesis (Table 2) where 102 unique metabolites were only obtained with the addition of the resins. These numbers clearly indicated that the real value of the addition of the resins to fungal fermentation on large sets of strains really consists in a general increment of the chemical diversity as an overall feature. However, the cause of these improvements remains unknown; they could be due to increased production beyond the detection threshold due to relief from feedback inhibition, relief from auto-toxicity, prevention of end product degradation, change on media composition by adsorption of certain components on the resins or other mechanisms. Moreover, and for some specific cases, the addition of the adsorptive polymeric resins could result even in larger changes in the SM profiles than the ones obtained

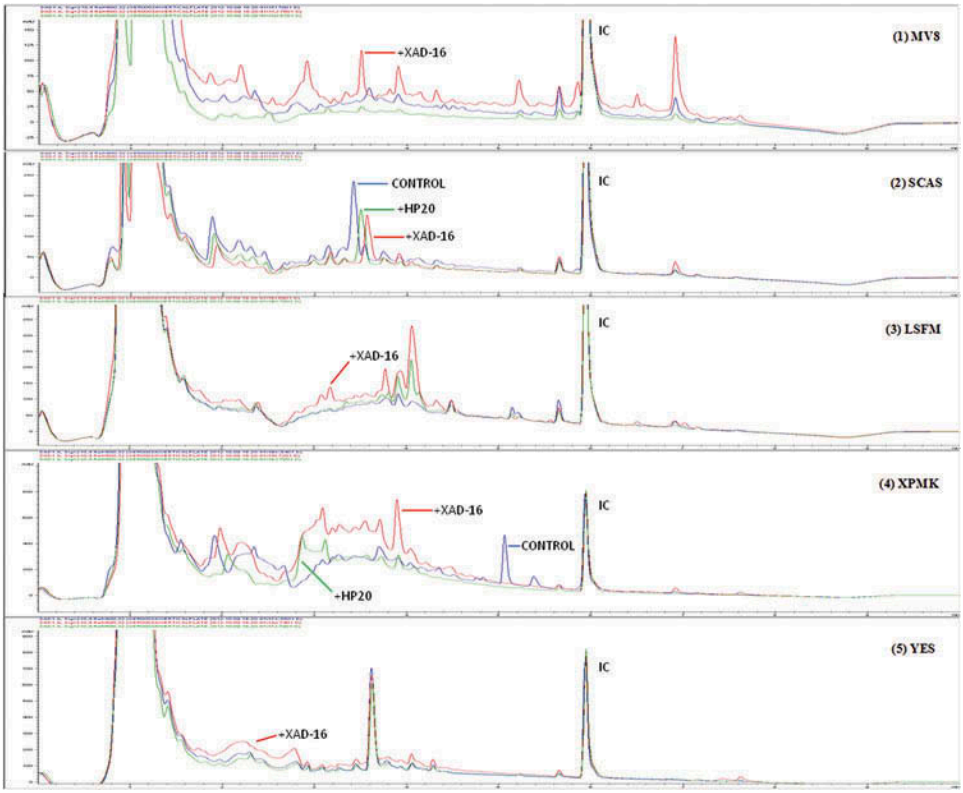

Figure 9. Comparative analysis of different uHPLC-UV 210 nm secondary metabolite profiles produced by strain *Preussia* sp. (CF-209171) when this fungus was grown on five different fermentation media (MV8, SCAS, LSFM, XPMK and YES) with and without XAD-16 and HP-20 resins. Relevant uHPLC traces are indicated in the figure. Internal control (IC) was added to each sample to allow accurate comparisons of the chromatographic runs.

by changing the composition of the base medium (Figure 10). These results clearly support the application of the adsorptive polymeric Amberlite® or Diaion® resins for specific strains where an increase in the production of specific metabolites or the generation of new bioactive metabolite cannot be achieved solely by introducing major changes in the medium components, e.g. addition of complex plant-based nutrients, or varying the carbon, nitrogen sources and the trace elements.

4. Conclusions

Several studies, on bacteria and fungi, have described the ability of adsorptive polymeric resins to increase the production titres of certain compounds of microbial origin (Frykman et al. 2006), and the possible mechanisms of action for these have been reviewed (Phillips et al. 2013). However, the question of whether one resin is consistently better than others for increasing metabolite chemical diversity has not been examined for any sets of strains. In order to evaluate this possibility, we selected the four most common resins used as microbial fermentation additives – Amberlite® XAD-2, XAD-7 and XAD-16, and Diaion® HP-20 – and we tested them in a pilot study for a set of 96 diverse fungal strains, grown in MMK2 fermentation medium, looking for increasing hit-rates of anti-infective activity and enhancements on metabolite production.

We were unable to correlate the additions of Amberlite® or Diaion® resins to the fermentation media of any fungal species with a direct increase in the production of non-cytotoxic antimicrobial metabolites for the widely diverse set of 96 fungal strains tested. Our bioactivity evaluation tests indicated that the addition of Diaion® HP-20 resin to the base fermentations medium MMK2 resulted in a decrease in the generation of cytotoxic and antimicrobial activities for these strains, whereas their fermentation in MMK2 medium, in the presence of Amberlite® XAD-2, slightly increased the frequency of detection of antibiotic and cytotoxic activity.

Analyses of their SM profiles indicated that variations in the composition of the base media increased the chemical diversity generated by the fungal strains to a larger extent than the addition of Amberlite® or Diaion® resins. It was also observed that variations on the end products of fungal fermentations in the presence of these polymeric resins could be related to an initial capture of essential nutrients by the resins or to a continuous action of the

Table 2. Number of unique secondary metabolites detected per microorganism and fermentation condition.

Strain ID	LSFM	LSFM +HP-20	LSFM +XAD-16	MV8	MV8 +HP-20	MV8 +XAD-16	SCAS	SCAS +HP-20	SCAS +XAD-16	XPMK	XPMK +HP-20	XPMK +XAD-16	YES	YES +HP-20	YES +XAD-16
CF-194989				*5*	*4*				*1*		*1*		*1*		
CF-195017	*1*	*1*	*2*						*3*		*1*			*1*	
CF-209155	*1*		*1*	*2*	*1*			*1*	*2*	*1*				*1*	*2*
CF-209171	*3*		*1*	*2*		*5*	*1*	*1*	*1*	*1*	*1*	*1*			
CF-209591	*2*		*2*	*3*		*6*		*1*	*4*	*1*	*1*	*1*	*1*		
CF-210345	*1*	*2*	*2*	*2*	*1*	*1*			*1*		*1*		*1*		*1*
CF-210367	*2*		*1*		*2*	*1*							*1*	*2*	
CF-210370						*1*	*1*			*1*					
CF-210988		*1*		*3*						*2*		*1*		*1*	*1*
CF-210989	*1*		*3*					*2*	*1*		*1*		*1*	*1*	*1*
CF-214546		*1*			*1*	*1*								*3*	*2*
CF-214552			*1*	*3*		*1*	*1*		*3*	*2*			*2*	*1*	
CF-214558	*1*		*1*			*1*	*1*		*3*		*1*		*1*		
CF-214575	*1*	*1*	*2*	*1*					*1*	*1*			*3*		*2*
Accumulated	*13*	*6*	*16*	*21*	*9*	*17*	*4*	*5*	*20*	*9*	*7*	*3*	*11*	*10*	*9*

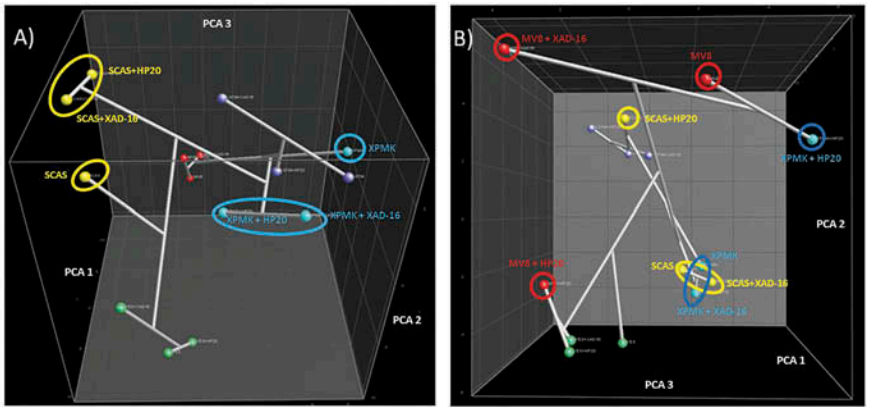

Figure 10. Principal component analysis (PCA) of the total of secondary metabolites produced by (A) *Pilidiella castaneicola* (CF-214558) and (B) *Preussia* sp. (CF-209171) grown in 15 different fermentation conditions. Relevant fermentation conditions are indicated in the figure.

resin throughout the whole fermentation process depending on the base medium composition.

Most of the fermentation conditions tested, either base medium changes, or additions of Amberlite® or Diaion® resins, produced unique compounds not found in any of the other conditions or induced changes in the production of specific compounds in most of the strains. Moreover, for certain strains, the addition of the adsorptive polymeric resins can result in greater changes in the SM profiles than changes obtained solely by varying the carbon, nitrogen and trace element composition. These findings might justify testing the addition of these resins when the production of a specific metabolite encoded by the fungal genome is desired.

In summary, this study is the first data-automated analysis on the effect of the addition of Diaion® and Amberlite® resins to fungal fermentations and their chemical diversity. Although changes in the medium composition more strongly influenced the SMs produced than the addition of adsorptive resins, the results clearly supported the hypothesis that the application of resins in fungal fermentations can determine the production of new SMs and provide a tool to consistently increase the production of low titre compounds. We believe these methods can be generally applied for the generation of natural product extract collections to get access to new bioactive chemical entities in drug discovery programs.

Acknowledgements

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Supplemental data

Supplemental data for this article can be accessed here.

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Chapter 3

Overview of the methods for purification of metabolites that are secreted by bacteria

3.1 INOCULATION AND INCUBATION OF BACTERIAL CULTURES

Literature pertaining to bacteriology

There are several sources that discuss the characteristics of bacteria that are pertinent to the growth of cultures in a laboratory. There are two types of bacteriology references known as the *Bergey's Manual*. Nine editions of the original, *Bergey's Manual of Determinative Bacteriology*, have been printed. The most recent of these was published in 1994 (Holt, Krieg, Sneath, Staley, & Williams, 1994). More recently, the *Bergey's Manual of Systematics of Archaea and Bacteria* was published. The current version of this source is only available in digital format on the Internet (Whitman, 2015). A reference book for choosing appropriate medium for the culture of bacteria has been published (Atlas, 2010) and two microbial knowledge bases may be accessed on the Internet (List_of_Prokaryotic_Names_with_Standing_in_Nomenclature, 2018; Strain_Info, 2018). A textbook designed to accompany a lecture course providing comprehensive coverage of the characteristics of bacteria and other microbes (Tortora, Funke, & Case, 2019) and a laboratory manual for a course in bacteriology (Johnson & Case, 2018) are also excellent references.

Choice of medium for the culture

A *complex medium* is one that includes an extract of tissue from an animal, plant, or fungus (Atlas, 2010; Johnson & Case, 2018; Tortora et al., 2019). Some examples of the components found in complex media are tryptone, peptone, and an extract

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of yeast. These media are considered to be complex because the exact chemical composition of such extracts is not known. Although most species of microbes grow well on one of the complex media, for any given species there are probably many substances in the complex medium that are not essential for growth.

In contrast, a *chemically defined medium* is one in which the exact concentration of each component is known. Only components that are either essential for the growth of the bacterium or necessary to modulate some aspect of the physiology for a particular experiment are included in such a medium.

There are at least two advantages to using a chemically defined medium for growing a culture of bacteria from which a secreted metabolite will be purified. The first is that this medium will be more specialized for the growth of the desired strain and therefore less likely to support the growth of contaminants. The second is that a defined medium is less likely to contain substances that may interfere with the structural characterization of the metabolite. For example, a chemically defined medium typically has several types of inorganic substances but only one type of organic substance. Complex media have many organic substances and it is the organic molecules that are most likely to interfere.

A recipe for a chemically defined medium that is known to allow growth of the desired bacterial strain, or a closely related strain, should be used. Liquid media in glass flasks are usually used unless the strain will only grow on agar medium. In addition to allowing for good growth of the strain, some other issues should be considered. The chosen medium should allow for secretion of the desired metabolite. Avoid using substances that are likely to give the medium color. If the metabolite that you plan to purify is fluorescent, do not use a medium that includes a fluorescent substance.

The starter culture and the subculture

To obtain a sufficient yield of the desired secondary metabolite, the volume of the bacterial culture from which the substance is purified should be between 0.5 and 2.0 L. The typical method for growing such a culture is as follows. A *starter culture* with a volume of 5.0−25.0 mL is inoculated with a single colony of the desired strain and then incubated until the culture is in exponential phase. A *subculture* of 0.5−2.0 L is then inoculated with a small aliquot of the starter culture. The subculture is

incubated until it is in exponential phase. If one inoculates the large culture directly from a colony on a plate instead of using this two-step method, there is a chance that the liquid culture will not grow and the procedure will have to be repeated with a fresh, large-volume batch of medium.

3.2 MONITORING THE GROWTH OF BACTERIAL CULTURES BY QUANTIFICATION OF TURBIDITY

Calculation of the cellular density of a culture from spectrophotometric data

The rate of growth of a bacterial culture may be quantified by measuring the increase in *turbidity* as the number of bacteria per milliliter increases (Koch, 1970). This quantity is sometimes called the *cellular density of the culture* and is measured with a *spectrophotometer*. Most spectrophotometers provide this measurement as a percentage of *transmittance*, *T*, or as a unitless *absorbance*, *A*. Visible radiation with a wavelength of 600.0 nm is typically used. Transmittance is denoted as T_{600} and absorbance as A_{600}. The use of the terms spectrophotometer, transmittance, and absorbance in chemistry for examination of molecular substances or ions in solution is explained in a later chapter in which absorption of radiation is discussed. For chemical analysis the relationship between these two measurements is given by Eq. (3.1) (Johnson & Case, 2018).

$$A = 2.0 - \log_{10} T \tag{3.1}$$

In microbiology, measurements of bacteria in a culture are described differently than are spectrophotometric measurements in chemistry. Although measurement of turbidity is shown as transmittance or absorbance on the display of the device, the magnitude of the absorbance is reported as the *optical density* or *OD* of the culture (Matlock, Beringer, Ash, Page, & Allen, 2011; Tortora et al., 2019). This is because visible radiation that contacts a bacterium is *scattered* so that none of it reaches the photometer (detector) of the device. In contrast, in chemical spectrophotometry only a portion of the radiation that contacts each molecule or ion is *absorbed*, while the remainder travels to the photometer. For microbiological assays Eq. (3.2) is used.

$$OD = 2.0 - \log_{10} T \tag{3.2}$$

Although turbidity may usually be quantified as either transmittance or optical density, in some cases it may be necessary to mathematically convert transmittance data to optical density data, or *vice versa*, after completion of the experiment. Eq. (3.2) is convenient for converting transmittance to *OD*, whereas a rearrangement [Eq. (3.3)] is more convenient for converting *OD* to transmittance.

$$T = 100.0 \times 10^{-OD} \tag{3.3}$$

If a culture is monitored by optical density, the following relation may be used for quantification. A culture of *Escherichia coli* bacteria with a cellular density of $1 \times 10^8\ \mathrm{mL}^{-1}$ has an OD_{600} of 0.6 (Winfrey, Rott, & Wortman, 1997). This observation may be used to derive Eq. (3.4) for calculating the density of cultures that have an OD_{600} less than or greater than 0.6.

$$\text{cellular density of culture } (\mathrm{mL}^{-1}) = \frac{1 \times 10^8}{10^{(0.6 - OD_{600})}} \tag{3.4}$$

One may find it more convenient, however, to monitor the culture by transmittance because Eq. (3.5), which is simpler, may then be used to calculate the density.

$$\text{cellular density of culture } (\mathrm{mL}^{-1}) = \frac{25.0\%}{T_{600}} \times 10^8 \tag{3.5}$$

A bacterium of *E. coli* has the shape of a rod that is 1.0 μm wide and 2.0 μm long (Tortora et al., 2019). These equations for calculation of the cellular density of a culture should be valid for species that are similar in size and shape to *E. coli*.

Dilution of an aliquot of the culture and correction for the dilution

Quantification of the OD_{600} of a bacterial culture is most accurate when the value reported by the spectrophotometer is between 0.1 and 1.0. This corresponds to values of T_{600} between 80.0% and 10.0%. If the OD_{600} that is measured is >1.0, and thus the T_{600} is $<10.0\%$, it is best to repeat the measurement with a diluted aliquot of the culture. The sample of culture should be diluted into fresh culture medium.

Two issues must be considered when correcting the spectrophotometric data with the dilution factor. The first is that the dilution decreases the OD_{600} whereas it increases the T_{600}. The second is that the relationship between cellular

density and OD_{600} involves an exponential function whereas the relationship between cellular density and T_{600} is linear. The two methods for correcting the data for the dilution are as follows:

- If T_{600} is measured, the value should be *divided* by the dilution factor to give the T_{600} of the culture prior to the dilution. This corrected T_{600} may then be placed in the appropriate equation.
- If OD_{600} is measured, do *not* apply the dilution factor directly to this value. Because of the exponential term in the equation that gives the cellular density from the OD_{600}, the dilution factor must be applied *after* the cellular density of the diluted sample is calculated. Calculate the cellular density of the diluted culture with the appropriate equation. To correct the calculated density of the diluted culture, *multiply* it by the dilution factor.

Calculation of the minimal generation time of a species of bacteria

Most species of bacteria reproduce by binary fission. If a culture is grown in ideal circumstances, the bacteria will reproduce as quickly as their physiology allows. Ideal circumstances include: the optimal temperature for growth, sufficient nutrients in the medium, the presence of molecular oxygen (if the strain requires it for optimal growth), and a large enough volume of medium to minimize inhibitory effects of bacterial waste. In such a case the density of the culture will increase exponentially. During a specified period of incubation the density will increase by a factor of 2^n, where n is the number of cellular fissions (generations) that occurred during that period (Tortora et al., 2019).

The time required for a bacterium to replicate its genome and divide to produce two daughter cells is the *generation time*. If the density of the culture is measured at two points in time while the culture is growing exponentially, and the number of generations calculated for this interval of time, the generation time may then be calculated. If the generation time for the strain of interest has been reported in the scientific literature, the value calculated for the strain currently being used may be compared to verify identity. It is important to consider the temperature at which the culture is being grown because rate of growth is very sensitive to temperature.

3.3 REMOVAL OF BACTERIA FROM A CULTURE BY CENTRIFUGATION AND FILTRATION

Sedimentation of the cells in a culture by centrifugation

In centrifugation, the test tubes or bottles that hold the solution of analyte are placed in a *rotor* and the center of the rotor is mounted on the *spindle*. Rotation of the spindle turns the rotor. For some centrifuges, there is more than one type of rotor that may be used. Sedimentation of cells is usually performed in a *swinging-bucket rotor*. In this type of rotor, the buckets that hold the test tubes or bottles are vertical before rotation begins, but reorient to a horizontal position after rotation starts.

The force exerted on the substances in the solution of analyte is known as the relative centrifugal force (RCF). The RCF is quantified as a multiple of the force of gravity, *g*. The equation that is used to calculate the RCF from the revolutions per minute (rpm) of the rotor may be found in the guide provided by the manufacturer of the centrifuge and rotor. The RCF is directly proportional to the distance from the center of the spindle. This distance is the *radius* and if swinging buckets are horizontal, the maximum radius is at the bottom of the bucket.

An example of the RCF in a swinging-bucket rotor that might be used for sedimentation of bacteria is as follows. If the radius to the bottom of each bucket is 20.9 cm, and it is revolving at 4200.0 rpm, the RCF at the bottom of the bucket will be 4122.0 $\times$ *g*. At least 90.0% of the bacteria in a culture will be sedimented by such a centrifugation. The supernate that includes the remaining bacteria is then decanted into a new container. Filtration may then be used to eliminate the residual bacteria.

Removal of the cells from a culture by filtration

A typical method for performing this type of filtration is as follows. A Fisherbrand circular filter that was purchased from Fisher Scientific is used. This filter is made of glass fiber, is of the G4 grade, and has a retention of 1.2 μm (a similar filter from another supplier may be used as an alternative). The filter is placed in a *Buchner funnel*, and the filter–funnel combination is placed in the neck of a *filtering flask* (Fig. 3.1). A filtering flask has a conical shape, the diameter of the opening at the top is smaller than the diameter of the base. It has a side arm (port) and is constructed of thicker glass than is found in a typical flask. This flask is used to create a vacuum underneath the

FIGURE 3.1 After centrifugation of the culture to remove the majority of the bacteria, filtration is used to remove residual bacteria from the supernate. *Reprinted with permission from Crowley, T. E., & Kyte, J. (2014).* Experiments in the purification and characterization of enzymes: A laboratory manual. *Amsterdam; Boston, MA: Elsevier/AP, Academic Press is an imprint of Elsevier. Retrieved from <https://lccn.loc.gov/2014395777.>.*

Buchner funnel. The thick glass prevents the flask from collapsing. Flexible tubing that will not collapse when a vacuum is applied to connect the side arm of the flask to an another filtering flask that functions as a *vacuum trap*. The trap is connected with tubing to a vacuum pump. The presence of the trap assures that no liquid will enter the pump.

The supernate is poured into the filter–funnel assembly and air is removed from the flasks by the pump. The vacuum drives the flow of supernate through the filter and the clarified liquid is collected in the flask that is directly below the funnel.

3.4 LIQUID–LIQUID EXTRACTION OF A METABOLITE FROM THE SUPERNATE OF A CULTURE

Two liquids are *immiscible* if they segregate into distinct layers when poured into the same container. The reason for this segregation is usually that one of the liquids is more polar than the other. The method in which a substance that has been dissolved in one solvent is transferred into another solvent is known as *solvent extraction* or *liquid–liquid extraction.*

To purify a secreted metabolite from an aqueous bacterial supernate, an organic solvent that has sufficient nonpolar character such that it is immiscible with the supernate is used. In the procedures described in this book, the organic solvent is

ethyl acetate. A *separatory funnel*, which has a removable glass plug in the round opening at the top and a rotating *stopcock* in the opening at the bottom, is used. This apparatus should be made of glass or other material that is resistant to the organic solvent that will be used.

Equal volumes of the supernate and the organic solvent are poured into the separatory funnel. The glass plug is inserted into the opening at the top and the funnel is shaken to intersperse the two liquids, thus creating an *emulsion*. The funnel is then mounted vertically on a ring stand. The emulsion segregates within a few minutes into two layers, the liquid having the greater density on the bottom. If the two liquids in the funnel are an aqueous solution and ethyl acetate, the aqueous solution will be on the bottom because the density of ethyl acetate is 0.90 g mL^{-1}. The stopcock is then opened for enough time to drain the liquid in the bottom layer into a beaker or flask. The liquid that is no longer needed is discarded. More extractions may then performed on the liquid that was saved.

3.5 SOLID-PHASE EXTRACTION OF A METABOLITE FROM THE SUPERNATE OF A CULTURE

An alternative to using ethyl acetate and liquid−liquid extraction for selection of a metabolite from a bacterial supernate is adsorption onto a solid phase. This technique is most commonly known as *solid-phase extraction* or SPE. The solid phase is also known as the *adsorbent*. Although elution of the metabolite from the adsorbent usually involves the use of an organic solvent, a solvent that is miscible with water may be used. An example of such a solvent is methanol. There are two advantages to using methanol rather than ethyl acetate for purification of a metabolite. One is that methanol is the smaller of the two molecules and therefore less likely to interfere with spectroscopic or spectrophotometric assays. The other is that ethyl acetate has an offensive odor, whereas methanol does not.

An example of an adsorbent that may be used for this method is beads of Amberlite XAD-2 from MilliporeSigma. This material is a hydrophobic copolymer of styrene-divinylbenzene resin. The adsorbent is dispersed into an aqueous supernate, thus creating a *slurry*. In this slurry, the nonpolar portion of molecules of a particular metabolite may bind to the beads. The bound substances may be released by immersing the adsorbent in a solvent such as methanol that is less polar than water.

Although the solvent of the eluant will be predominantly the organic liquid, there will also be some water. It is best to minimize the amount of water because it is less volatile than most organic liquids, and therefore harder to remove in the evaporation procedure that is discussed in a later section in this chapter.

The method for minimizing water in the eluant is as follows. The mixture of aqueous supernate and adsorbent is transferred to a cylindrical tube that is positioned vertically and has a stopcock at one end (Fig. 3.2). This creates a *chromatographic column*. The method to be used in this case is not chromatography; however, the use of the column arrangement provides greater control as the solvent is changed from aqueous to organic. The excess aqueous solution is allowed to drip out of the column and then the organic solvent is poured into the top of the column. As the organic solvent drips out of the column, it carries with it the metabolite that has been released from the adsorbent.

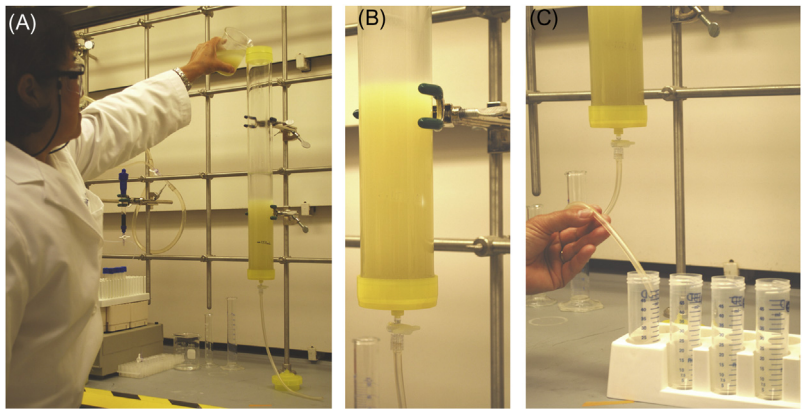

FIGURE 3.2 A chromatographic column is used to elute the bound metabolite from the adsorbent. (A) While the stopcock at the bottom of the column is closed, the aqueous slurry of adsorbent, with bound metabolite, is poured into the column. (B) The beads are allowed to settle to the bottom. (C) The stopcock is opened for enough time to allow the excess aqueous solution to drip out of the column. The aqueous solution is discarded. The organic solvent is then poured into the column and the stopcock is opened for enough time to allow this liquid to drip out of the column. This eluant contains the metabolite that has been released from the adsorbent. It is not necessary to use an automated fraction collector. *Reprinted with permission from Crowley, T. E., & Kyte, J. (2014).* Experiments in the purification and characterization of enzymes: A laboratory manual*. Amsterdam; Boston, MA: Elsevier/AP, Academic Press is an imprint of Elsevier. Retrieved from <https://lccn.loc.gov/2014395777.>.*

3.6 DRYING, RESUSPENSION, QUANTIFICATION OF YIELD AND STORAGE OF A PURIFIED METABOLITE

Drying

The organic solvent in which the metabolite is dissolved, after liquid–liquid extraction from the supernate of the culture or elution from the adsorbent after SPE from the supernate, may be removed by evaporation. There are two reasons for drying the preparation. The first is that the metabolite may then be resuspended in a smaller volume, providing a more concentrated solution of this substance. The second is the option of using a different solvent for resuspension than was used for extraction or elution.

Removal of water associated with an organic solvent that is not miscible with water

If the solvent that was used for extraction or elution is not miscible with water, and is less dense than water (e.g., ethyl acetate), the following will probably be observed. A small volume of water from the supernate ($\sim$1.0% of the volume of the organic extract) may be present at the bottom of the container, underneath the organic liquid. Because the two liquids are immiscible, the water will appear to be a bubble trapped between the wall of the container and the organic liquid. Because all of the metabolite is expected to be in the organic liquid, and water evaporates more slowly than most of these solvents, it is best to eliminate the water before beginning the process of evaporation.

The water may be removed by adding anhydrous magnesium sulfate to the container. This inorganic salt will sink to the bottom and adsorb the water. The organic liquid, which contains the metabolite, may then be decanted into another container. This new container, as described below, should be suitable for evaporation. Evaporation of the organic solvent may then be conducted as described below.

Removal of water that is mixed with an organic solvent

If the organic liquid that was used for extraction or elution is miscible with water (e.g., methanol), then any water in the preparation cannot be removed with magnesium sulfate. The water has to be removed by the same process by which the organic solvent is removed, that is, evaporation. Water is

less volatile than most organic liquids. Even if the water represents only 1.0% of the total volume, its presence will probably increase the time required for the evaporation to be completed.

Evaporation of the organic solvent

The organic liquid in which the metabolite is dissolved should be poured into a beaker. The diameter of the opening at the top of a beaker is the same as the diameter of the base. Do not use a container in which the opening at the top has a smaller diameter than the diameter of the base (e.g., an Erlenmeyer flask). Evaporation is very slow in this type of flask.

Evaporation should be conducted in a fume hood. In a hood, there is a flow of air across the open beaker and upward toward the exhaust port. This is helpful for two reasons. The first is that it prevents the workers in the laboratory from being exposed to toxic vapors. The second is that it accelerates the process of evaporation.

The dimensions of the beaker used to initiate evaporation should allow for rapid evaporation of the solvent. For the exercises in this book, the volume of the first beaker should be between 1.0 and 4.0 L. As the volume of the liquid decreases, it may sequentially be transferred to smaller beakers. A beaker with a volume of 50.0 mL is appropriate for the final step in which the last portion of liquid evaporates, leaving the residue that includes the dry metabolite.

Resuspension

If a metabolite is soluble in water, and in one or more organic solvents, there is an advantage to resuspending it in an organic liquid rather than water. Most organic liquids are more volatile than water [dimethyl sulfoxide (DMSO) is an exception as explained below]. At some time after the dry metabolite is resuspended in the solvent, it may be necessary to prepare a solution of the metabolite in a different liquid. An organic solvent may be removed by evaporation more rapidly than water may be removed. The dry metabolite may then be resuspended in the alternative solvent.

Although DMSO is suggested as the solvent for samples to be examined with nuclear magnetic resonance spectroscopy in exercises in this book, it is not recommended as the solvent for resuspension of the entire preparation of the metabolite. The boiling point of this organic liquid under atmospheric pressure

is 189.0°C. If the metabolite is dissolved in DMSO, it is difficult to remove this solvent.

To determine the appropriate volume of solvent in which to resuspend the dry metabolite, consider the limit of solubility and the minimum concentration needed for the assays that will eventually be performed. An example of the typical range of concentrations is as follows. In the exercises presented in later chapters in which detailed protocols are provided for purification of metabolites, the molar masses of the substances purified will probably be between 200.0 and 600.0 g mol^{-1}. The molar yield of these molecules will probably range from 10.0 to 80.0 μm, corresponding to a mass yield of 4.0−18.0 mg. If the purified metabolites are resuspended in the recommended volumes, that is, from 2.0 to 8.0 mL, then the molar concentration of the substances will be between 2.0 and 10.0 mM, corresponding to a mass per volume concentration of 1.0−2.0 mg mL^{-1}.

Quantification of yield

Assay of the absorbance of ultraviolet (UV) or visible radiation by a solution of the purified metabolite followed by some calculations will reveal the concentration of this substance in the resuspended sample that was described above. This calculation is explained in the later chapter that focuses on absorption of radiation. The concentration of the metabolite, and the volume in which the substance was resuspended, may then be used to calculate the yield.

Storage of a purified metabolite

If the metabolite is dissolved in an organic solvent, the test tubes used for storage must be constructed of material that is resistant to the solvent. The resuspended sample should be divided into several portions and each stored in a distinct test tube. If the preparation is divided in this way, only a portion of it is exposed to the atmosphere when a aliquot is removed for an experiment. To prevent evaporation of the solvent, the caps should be tightly sealed. To prevent photolysis of the metabolite, the test tubes should be shielded from UV and visible radiation. If a refrigerator or freezer is available, storage at the lower temperature will minimize the possibility of microbial growth in the samples. Although some metabolites may be stable at the typical ambient temperature in a laboratory

(22.0°C), storage at the lower temperature may also prevent degradation of the purified substance.

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AMBERLITE® XAD16

Industrial Grade Polymeric Adsorbent

PRODUCT DATA SHEET

AMBERLITE XAD16 is a polymeric adsorbent supplied as insoluble white beads. It is a nonionic, hydrophobic, crosslinked polymer which derives its adsorptive properties from its patented macroreticular structure (containing both a continuous polymer phase and a continuous pore phase), high surface area, and the aromatic nature of its surface (see figure 1). AMBERLITE XAD16 polymeric adsorbent issued to adsorb hydrophobic molecules from polar solvents and volatile organic compounds from vapor streams. Its characteristic pore size distribution makes AMBERLITE XAD16 polymeric adsorbent an excellent choice for the adsorption of organic substances of relatively low to medium molecular weight. It can be used in column or batch operations.

PROPERTIES

Matrix	Macroreticular aliphatic crosslinked polymer
Physical form	White translucent beads
Moisture holding capacity [1]	62 to 70 %
Shipping weight	720 g/L
Specific gravity	1.015 to 1.025
Particle size	
Harmonic mean size	0.56 - 0.71 mm
Uniformity coefficient	≤ 2.0
Fines content [1]	< 0.350 mm : 2.0 % max
Coarse beads	> 1.18 mm : 2.0 % max.
Maximum reversible swelling	see Table 1
Surface area [2]	≥ 800 m^2/g
Porosity [2]	≥ 0.55 ml/ml

[1] *Contractual value*

[2] *Values based on statistical quality control (SQC)*

Test methods are available on request

SUGGESTED OPERATING CONDITIONS

pH range	0 - 14
Maximum temperature limit	150°C
Minimum bed depth	75 cm (Capture)
Flow rate	
Loading	2 to 16 BV*/h
Displacement	1 to 4 BV/h
Regeneration	1to 4 BV/h
Rinse	2 to 16 BV/h

** BV (Bed Volume) = 1 m^3 solution per m^3 resin*

PROPERTIES (CONTD.)

Figure 1 : Chemical structure of AMBERLITE XAD16 polymeric adsorbent

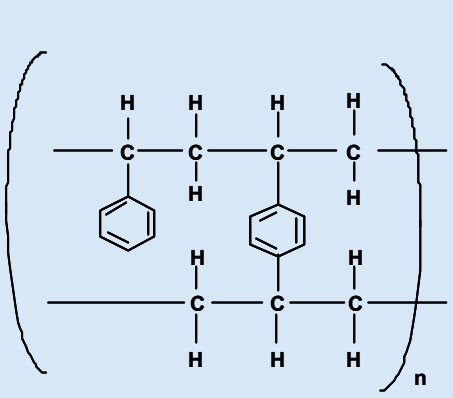


Figure 2 : Pore distribution of AMBERLITE XAD16 polymeric adsorbent

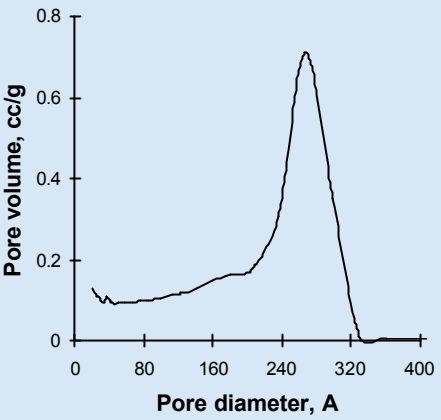


Figure 3 : Infrared Spectrum of Amberlite XAD16 polymeric adsorbent

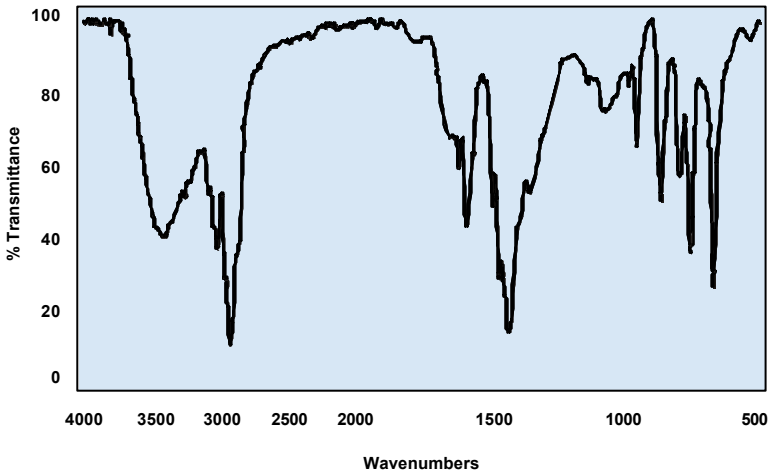


Table 1: Percent swelling of Amberlite XAD16 polymeric adsorbent in various solvents (Water: Solvent)

Solvent	% Increase from as-received volume
Methanol	15
2-propanol	15
Acetone	20
p-Xylene (via methanol)	25

PRETREATMENT

AMBERLITE XAD16 polymeric adsorbent is shipped as a water wet product imbibed with sodium chloride (NaCl) and sodium carbonate (Na_2CO_3) salts to retard bacterial growth. These salts must be washed from the adsorbent prior to use and it is suggested that this be achieved by washing with water at a linear flowrate of 5-10 m/h until the required level is achieved. In some sensitive applications, residual monomeric or oligomeric compounds may be required to be removed from the adsorbent. A regeneration with the proposed regenerant is also recommended prior to beginning the first service cycle. If the regenerant is an alcohol, it must be displaced with water prior to beginning the first loading cycle.

SAMPLE PREPARATION FOR TESTING

Samples of Amberlite XAD16 polymeric adsorbent must be pre-treated prior to laboratory testing to ensure proper results. Please refer to Rohm and Haas publication IE-245 "Laboratory Column Procedures and Testing of Amberlite and Duolite Polymeric Adsorbents", section "Preparation of Resins".

APPLICATIONS

- **Recovery and purification of antibiotics, water soluble steroids, enzymes , amino acids and proteins**.
  AMBERLITE XAD16 can be considered as a general purpose resin for these types of applications combining good mesoporosity with high surface area. In these types of applications, of which the recovery of Cephalosporin C is perhaps the best example, the loading and elution flowrates are relatively low (0.5-2 BV/h). The pH of the solution has a significant effect on the loading and elution and as the feed is often derived from a fermentation, the regeneration tends to be aggressive - 4% NaOH at elevated temperatures and solvents. A primary concern in this type of application is the separation of two or more similar solutes. In these cases, the engineering is a key point to consider during both scale and final plant design.

- **Removal of non polar compounds, such as phenol, from polar solvents**.
  These types of applications can be considered a simple capture step where the adsorbent resin is used to remove a small number of solutes from a process stream, often a waste stream. AMBERLITE XAD16 will prove useful in this type of application where the size of the solute is relatively large (> 200 D) and where the operating capacity on AMBERLITE XAD4 may be lower.

- **Fruit juice upgrading**.
  For this application, AMBERLITE XAD16HP is specifically recommended.

REGENERANTS / ELUTING AGENTS

- Water miscible organic solvents (methanol, ethanol, acetone, isopropanol, etc.) for hydrophobic compounds.
- Pure solvents for regenerating resin fouled by oils and antifoams.
- Dilute bases (0.1 - 0.5% NaOH) for eluting weakly acidic compounds.
- Concentrated bases (2-4% NaOH) for regenerating resins fouled with proteins, peptides.
- Dilute acids (0.1 - 0.5% HCl) for weakly basic compounds.
- Dilute oxidising agents (< 0.5%) such as peroxide to enhance the removal of protein fouling.
- Buffer elution for pH sensitive compounds
- Water where adsorption is from an ionic solution.
- Hot nitrogen or steam for volatile materials.

FDA CLEARANCE

- Amberlite XAD16 polymeric adsorbent has clearance under FDA Food Additive Regulation 21CFR173.65-Divinylbenzene Copolymer. The product may be used for the removal of organic substances from aqueous foods under the prescribed conditions outlined in 21CFR173.65.

HYDRAULIC CHARACTERISTICS

Figure 4 shows the bed expansion of AMBERLITE XAD16 as a function of backwash flow rate and water temperature. Figure 5 shows the pressure drop for AMBERLITE XAD16, as a function of service flow rate and water temperature. Pressure drop data are valid at the start of the service run with a clear water and a correctly classified bed.

Figure 4 : Bed Expansion

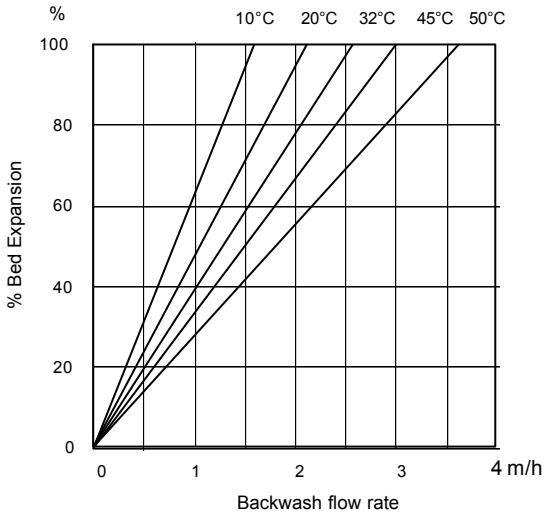
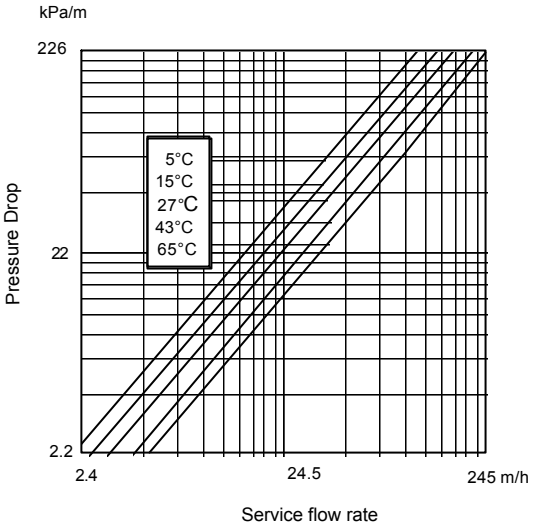


Figure 5 : Pressure Drop



Material Safety Data Sheets

Material Safety Data Sheets (MSDS) are available for all Amberlite polymeric adsorbents. These sheets contain pertinent information that you may need to protect your employees and customers against any known health or safety hazards associated with our products.

We recommend that you obtain copies of our MSDS from your local Rohm and Haas technical representative before using our products in your facilities. We also suggest that you contact your suppliers of other materials recommended for use with our products for appropriate health and safety precautions before using them.

Rohm and Haas/Ion Exchange Resins - Philadelphia, PA - Tel. (800) RH AMBER - Fax: (1)-215-409-4534
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Solid-Phase Extraction (SPE)—a Comparison of 16 Materials for the Purification of Anthocyanins from *Aronia melanocarpa* var Nero

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Abstract: There is an increasing interest in anthocyanins, not only as natural food colourants but also for pharmaceutical products due to their antioxidative potential. Common extraction procedures of anthocyanins from plant material are non-selective and yield pigment solutions with large amounts of by-products such as sugars, sugar alcohols, organic acids, amino acids and proteins. Some of these impurities may accelerate anthocyanin degradation or cause problems in further processing steps such as spray drying. In order to obtain a highly concentrated and purified anthocyanin pigment from black chokeberry (*Aronia melanocarpa* var Nero), juices and skin extracts were purified by solid-phase extraction (SPE). Sixteen solid-phase materials were tested on a laboratory scale, whereby the anthocyanin and sugar content of collected fractions were determined to represent elution profiles. Among these, reversed-phase silica gels and macroreticular non-ionic acrylic polymer adsorbents such as Serdolit PAD IV or Amberlite XAD-7 turned out to be most suitable. SPE was investigated with these materials in an enlarged scale, improving elution gradient and column purification. Amberlite XAD-7 was successfully applied in a 36-litre-scale separation.

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Key words: *Aronia melanocarpa*; black chokeberry; anthocyanin; food colourant; purification; solid-phase extraction

INTRODUCTION

Anthocyanins are probably the best known natural food pigments, since they impart red and blue colours to many fruits and vegetables. Their commercial rarity and difficulty in purification from natural sources, as well as the fair stability of anthocyanins which is restricted to acidic pH values, are some of the reasons limiting their application as food colours. Yet, they give an attractive red colour to many fruit juices, soft drinks, wines, jellies and jams etc.

Aronia melanocarpa (Michx) Elliott (black chokeberry) is a suitable and rather cheap source of a red to violet coloured pigment. It contains the anthocyanins cyanidine 3-*O* galactose, cyanidine 3-*O* arabinose, cyanidine 3-*O* glucose and cyanidine 3-*O* xylose (Oszmianski and Sapis 1988). About three-quarters of the pigments are retained in the fruit skin, which is therefore taken for extraction (Plocharski and Zbroszczyk 1992). *A melanocarpa* has up to 26% sugars, with a high content of sorbitol. Further components are organic acids (mainly malic and tartaric acid, as well as citric and succinic acid), flavonoids, vitamins (C, B2, B9,

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E, K1, nicotinic and folic acid, riboflavin, and provitamin A) and flavour compounds, the concentrations of which are dependent on the maturation status of the berries (Strigl *et al* 1995a).

Anthocyanins are generally generated either directly by concentration of coloured fruit juices or by alcoholic extraction of the plant material. As the extraction procedure is not selective, a number of other plant constituents may be co-extracted along with the desired pigments. In the case of *Aronia melanocarpa*, glucose, fructose and sorbitol are the most plentiful and troublesome 'impurities' in the pigment extract. They lead to Maillard type browning reactions, and their decomposition products may increase the anthocyanin degradation rate. Furthermore, they complicate the following processing steps, eg spray drying. The presence of amino acids accelerates the rate of pigment degradation as well (Chiriboga and Francis 1973).

The purpose of the present work was to study some technology aspects related to the purification of anthocyanins from *Aronia* juice and *Aronia* concentrate. For pharmaceutical products and some food-grade colouring additives, a defined purity is required. Therefore, adsorption–desorption methods based on the use of selective adsorbents and eluents were used.

Purification by solid-phase extraction (SPE) is a relatively simple method allowing the elimination of polar, non-phenolic impurities in one step. In order to maintain the selectivity of the stationary phase and eluant, a reasonable lifetime is necessary, such that the interactive surface of the media remains constant. Changes in the selectivity of the surface result from chemical degradation and/or chemical fouling. Chemical fouling occurs when components in the process stream (either product or impurities) are irreversibly bonded to the chromatographic surface and thereby alter its selectivity. This type of fouling occurs with polymerised anthocyanins which are present in aged pigments. In many instances, this also results in a reduction of the capacity of the stationary phase. The material, the elution and regeneration steps, therefore, should be optimised in order to insure complete recovery of both the product and the impurities. Cycle-to-cycle reproducibility and good stationary-phase lifetime should be achieved. A specification for the composition of the eluting solvents should be established. It is necessary to generate the appropriate gradient in a reproducible manner.

In the initial laboratory study presented here, the chromatographic variables for individual steps are carefully optimised in order to maximise the purity, consistency and throughput of the product. Problems that may arise are losses due to irreversible adsorption, an incomplete removal of contaminants or the contamination of eluates with oligomeric extractives from the adsorbents (Andersen 1988).

The procedure of SPE involves application of juices and crude aqueous extracts of anthocyanins after filtration or centrifugation to a column and elution with alcoholic solvents. Acidified methanol or ethanol are suitable for the elution of anthocyanins from polymeric adsorbents. Acidification is necessary to prevent anthocyanin oxidation, causing their instability in neutral and alkaline pH (Lee 1992). With appropriate solvents and solid-phase materials, a separation of individual anthocyanin components is also possible (Strack and Wray 1989).

Methods of column separation are proposed as a first step for the isolation of anthocyanins on a moderate scale (Hendry and Houghton 1992; Lalaguna 1993). In the present work, the authors intended to develop a purification procedure for application in an enlarged-scale process.

MATERIALS AND METHODS

Raw material

Aronia melanocarpa var Nero was obtained from plantations in Slovakia. SPE was carried out with juice of black chokeberry as well as with alcoholic and aqueous skin extracts with and without SO_2. To generate juice, *A melanocarpa* was mixed in a blender (Krups ProMix 170 metal) for 4 min. The mash was centrifuged for 30 min (Haermle-Laborzentrifuge, 2260 g) and the juice obtained was used for SPE (Fuchs *et al* 1996). Aqueous extracts were gained by dilution of pigment concentrates, which were obtained by ethanolic extraction of *A melanocarpa* pomace (Plocharski and Zbroszczyk 1992).

Preparation of solid-phase material and chromatography

The solid-phase materials were soaked in water or diluted ethanol, and undersized particles were removed with the supernatant. For the preliminary screening experiments, slurry was packed in glass tubes with Teflon filters (Lichrolut 8 ml, fill volume 5–6 ml). After filling, the resins were washed with 50 ml of bidistilled water. Fractogel TSK CM 650 (S) (Merck), a weakly acidic cation exchanger, was rinsed with HCl, until Na^+ was exchanged to H^+. With Lewatit MP 64 (Bayer), the OH^- was exchanged against Cl^- using NaCl/HCl.

For the initial experiments, 0·5 ml of aqueous coloured liquid was applied to each column. Water-soluble impurities were washed out by rinsing the column with distilled water until no glucose could be detected with a glucose kit (S-Glukotest, Art. NR. 126152, Boehringer Mannheim, Germany). Afterwards, the adsorbed pigments were eluted by increasing concentrations of ethanol acidified with H_2SO_4 or citric acid. The dense part of the anthocyanin band was collected and concentrated at 50°C *in vacuo* for recovery and re-use of the acidified ethanol. Silica gel RP-C 18 (Fluka), Serdolit

PAD IV (Serva) and Amberlite XAD-7 (Sigma) were tested out in an enlarged scale in self-filled glass tubes of 20 ml volume. After each run, the latter materials were purified with acidic 96% ethanol and re-used for 5–6 further experiments.

With Amberlite XAD-7, a scale-up experiment in a pilot-plant column of 16·5 × 170 cm size (bed volume 36 litres) was carried out, applying the juice and skin extracts of about 100 kg of *A melanocarpa*.

Analytical procedures

In the laboratory scale, fractions of 1–5 ml were taken and weighed. Density was measured by a Paar DMA 48 densitometer. Sugars (glucose, fructose and sorbitol) and acids were determined with HPLC (Column HPX-87H, eluent 0·01 M H_2SO_4, flow 0·45 ml min^{-1}, temperature 60°C, Biorad RI detector model 1755, integrator HP3394A). Colour Units were measured after Strigl *et al* (1995b) at pH 1 in an Al^{3+} buffer at 512 nm with a UV/Vis spectrophotometer (Perkin Elmer, Lambda 1). From the Colour Units, the anthocyanin concentration (g kg^{-1}) can directly be calculated by multiplication with the molecular weight (445·2 for cyanidine 3-*O* glucose or galactose) and division by the molar extinction coefficient $\varepsilon = 26\,900$ which was estimated for cyanidine 3-*O* glucose in an aqueous buffer system at pH 1 (Jurd and Asen 1966). Parameters describing the quality of anthocyanin colour such as browning, polymeric colour and degradation index were determined after Wrolstad (1976).

HPLC analysis of anthocyanins was carried out after a modified method of Koswig and Hofsommer (1995) (Waters millipore Chromatography LC-Module I with millenium 2010 software, UV detection at 518 nm, column RP-18 (5-µm particles Merck); flow 1 ml min^{-1}; internal standard, cyanidincloride, Rothe 4545·4 for HPLC).

RESULTS AND DISCUSSION

Sixteen solid-phase materials separating by different kind of modes were chosen (Table 1), with emphasis being placed on materials proposed in the literature for the purification of anthocyanins and polyphenols. Among these are non-polar reversed-phase materials, non-ionic polymeric adsorbents and weak and strong ionic exchange resins. In most cases, the mode of interaction between pigment and solid phase is not exactly known.

In the 8 ml scale, the best results were achieved with reversed-phase silica gels, especially RP-C18, followed

TABLE 1
Materials tested for solid-phase extraction of *Aronia melanocarpa* anthocyanin solutions

Material	*Specification*	*Mode of separation*	*Comment*
Amberlite XAD-2	Fluka, 20–50 mesh	Non-ionic, polyaromatic adsorbant resin	Insufficient anthocyanin retention, pigment is eluted with water
Amberlite XAD-7	Fluka, 20–50 mesh	Non-ionic, polyaromatic adsorbant resin, higher specific surface	Poor separation, insufficient anthocyanin retention, column difficult to clean
Amberlite XAD-7	Sigma, 20–60 mesh	Non-ionic acrylic ester resin moderate polarity	Suitable, good separation, cleaning of material with 95% strongly acidic ethanol
Serdolit PAD IV	Serva, 0·3–1·0 mm	Non-ionic acrylic ester/divinyl benzene copolymer	Good separation, suitable
Sephadex G-25	Pharmacia Fine Chemicals	Gelfiltration	Anthocyanins are fixed to the column
PVPP	Sigma	Binding of phenolic moieties	Anthocyanins are fixed to the column
RP Silica gel 100, C-18	Fluka, 230–400 mesh	Reversed phase, non polar	Very good separation concentration of anthocyanins, rather easy cleaning of column material
RP silica gel 60	Merck, 30–70 mesh	Reversed phase, non polar	Suitable separation, easy cleaning of column material
silica gel	Merck, 30–70 mesh	Strongly polar	No separation of sugars and anthocyanin
DEAE-Cellulose	Serva	Anion exchanger	Fair separation, losses of colour pigments
DEAE-Sephadex	Pharmacia Fine Chemicals, 40–120 µm	Anion exchanger	Poor separation, high losses of colour
Lewatit MP 64	Bayer	Basic anion exchanger, (Cl^-)	High colour losses due to alkaline pH
Fractogel TSK CM 60	Merck, 0·025–0·05 mm	Weakly acidic cation exchanger (Na^+)	Good separation, pH too high for anthocyanins, vacuum necessary for flow
Fractogel TSK CM 650	Merck, 0·025–0·05 mm	Weakly acidic cation exchanger (H^+)	Very good separation, vacuum necessary for flow because of small particle size
Amberlite CG-50	Aldrich, 100–200 mesh	Weakly acidic cation exchanger	Good separation of sugars, column difficult to clean
Amberlite IRC-50	Serva, 20–50 mesh	Weakly acidic cation exchanger (H^+) on polystyrene	Poor anthocyanin retention, colour is eluted with water
Dowex 50 WX8	Aldrich, 50–100 dry mesh	Strongly acidic cation exchanger (H^+) on polystyrene	Poor separation, column hard to clean

by Amberlite XAD-7, Serdolit PAD IV and Fractogel TSK CM 650 (H^+). With these materials, the overall colour losses were negligible ($\leqslant 5\%$) and the anthocyanin quality expressed by spectroscopic parameters such as degradation index, browning and polymeric colour (Wrolstad 1976) remained unchanged. Water-soluble organic and inorganic impurities could be removed by washing with water (Table 1), while the anthocyanins were bonded tightly enough to withstand aqueous elution. With these materials, separation of anthocyanins from sugars, sorbitol and acids was possible in juice and aqueous extracts with or without SO_2 as well. Figures 1–4 depict elution profiles of *Aronia* anthocyanins and sugars from suitable solid phases. By measuring the density and pH of the effluent water, the removal of sugars and acids is indicated in a simple

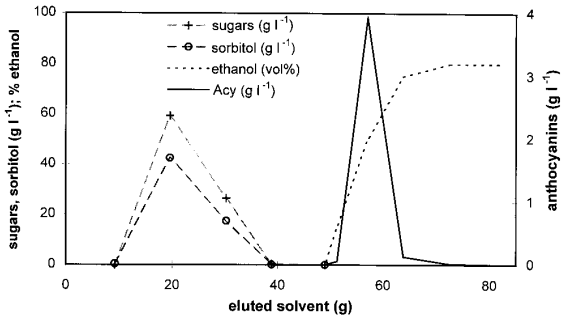

Fig 1. Solid-phase extraction of *Aronia* juice with silica gel RP-C18; 20 ml scale; application, 10 ml juice with 2·2 g litre^{-1} anthocyanins (Acy).

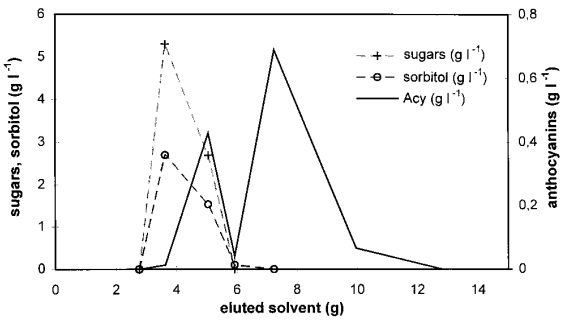

Fig 2. Solid-phase extraction of ethanolic *Aronia* skin extract with silica gel RP-C18; 8 ml scale; application, 0·5 ml extract with 3·9 g litre^{-1} anthocyanins (Acy).

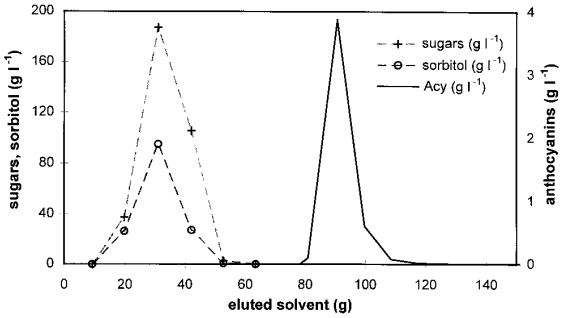

Fig 3. Solid-phase extraction of *Aronia* juice with Amberlite XAD-7; 20 ml scale; application, 20 ml juice with 2·2 g litre^{-1} anthocyanins (Acy).

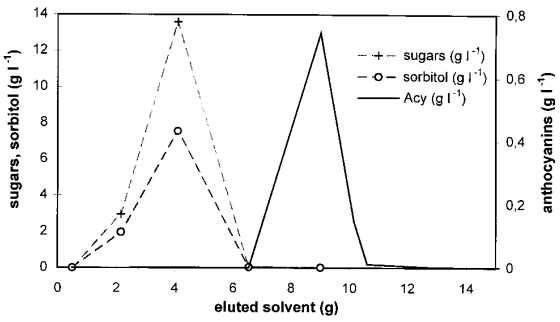

Fig 4. Solid-phase extraction of *Aronia* juice with Fractogel TSK CM 650 (H^+); 8 ml scale; application, 0·5 ml juice with 1·9 g litre^{-1} anthocyanins (Acy).

way. Yet, applying ethanolic colour extracts, the anthocyanin retention was incomplete. The colour pigments came in two separated peaks, the first one at the same time as the sugars (Fig 2).

With silica gel RP-C 18, the anthocyanins are eluted from the column as a narrow band in front of the ethanol, when the washing water is replaced by the eluent. The treatment gives a highly concentrated anthocyanin solution. In most cases, a small band of reddish-brown coloured pigments remained at the top of the column and did not elute even with 96% ethanol. This is more likely to be higher polymerised anthocyanins than aglycones. Anthocyanidins which are degradation products possibly arising from acidic hydrolysis of sugar moieties are less polar than anthocyanins and therefore more tightly bound to a non-polar matrix (Van de Casteele *et al* 1983). Thus, glycosides are already eluted in aqueous ethanol, while 95% ethanol should suffice also for the elution of aglycones. Highly polymerised anthocyanin degradation products, however, are insoluble in water as well as in alcohol (Fuleki and Francis 1968).

With Amberlite XAD-7, separation and recovery was excellent, but the coloured fractions were not as concentrated as with RP-C 18. With Serdolit PAD IV, a similar product, approximately the same results were obtained. However, with the technical grade of the latter material that was used, impurities from the solid phase were co-eluted with the pigments.

Fractogel TSK CM 650 in the Na^+ form turned out to be too alkaline for anthocyanin stability. This was also valid for the anion exchange resins, where the eluates had pH values above 5 at the start. In general, acidification of the alcoholic eluent and probably also of the washing water leads to a better anthocyanin recovery. Elution without acidification caused a red-to-violet colour change on the column, because of anthocyanin degradation to the quinoidal form. Finally, anthocyanin elution is incomplete with neutral or slightly acidic solvents. For acidification, organic acids such as citric acid are preferred in the food industry. Citric acid is less corrosive, acts as chelator for metal ions and thus may exert a protective effect on antho-

cyanins throughout further processing (Timberlake and Bridle 1980). However, for the final purification of many solid phases such as Amberlite XAD-7, concentrated ethanol acidified with strong inorganic acids such as H_2SO_4 is necessary.

Owing to the wide polarity scale of anthocyanins, gradient elution has usually been performed in most published studies (Goiffon, *et al* 1991). This may lead to several fractions with a different anthocyanin pattern. As the aim was not the separation of individual anthocyanins, it was found that elution is faster and sharper fractions are obtained by direct application of 70–80% ethanol.

Among the other materials tested, some bind anthocyanins very tightly so that either the need of solvent for their elution is uneconomically high or there are losses due to irreversible adsorption. This is especially the case for Sephadex G-25 and Polyvinylpolypyrrolidon (PVPP) but also for the ion exchangers Dowex WX8, Amberlite CG-50, DEAE-Sephadex and DEAE-Cellulose. PVPP tends to form unusually strong hydrogen bonds with the hydrogen of phenolic hydroxyl groups, due to the presence of oxygen in the peptide linkage. Extensive diffusion of the colour on the column and some pigment degradation were observed. PVPP can be applied for the removal of phenolic compounds during the isolation of plant protein extracts (Jackman *et al*1987). It is effective for the elimination of anthocyanins in fruit juices prior to HPLC analysis of sugars, as phenolic compounds can shorten the lifetime of some columns.

Whilst in the literature (Fuleki and Francis 1968) Amberlite CG-50 is most frequently proposed for purification of anthocyanins, it was found that anthocyanins were bound too tightly for the target of a quick purification step.

On the contrary, with Amberlite IRC-50, a weakly acidic cation exchanger, the colour pigments diffuse into the column and the anthocyanins are eluted together with sugars. Purification with Amberlite XAD-2 and Amberlite XAD-4, non-polar copolymers of styrene-divinylbenzene, was not satisfactory. While acrylic ester type resins were most appropriate, the polyaromatic non-ionic resins were not found to be suitable for the retention of anthocyanins. With XAD-2, the pigments washed out with the sugar; with XAD-4, it was not possible to get all colour pigments from the column (Table 1 lists the results of all tested materials).

Scale-up experiments were carried out with Amberlite XAD-7 and silica gel RP-C18 in a 20 ml scale by doubling the amount of the applied chokeberry extract. As a result, it was found that the conditions of separation and colour concentration in the eluate are best near the maximum load. Colour losses are then about 1%, while glucose, fructose and sorbitol are completely removed. The anthocyanin composition did not change during the purification step, and quality parameters such as browning, polymeric colour or degradation index remained constant (data not shown). By measuring the breakthrough curves through overloading, the capacity of the column was estimated to be about 13–14 mg of anthocyanins per ml of column material for the used RP-C18 and about 7 mg anthocyanins per ml of Amberlite XAD-7. It should be borne in mind that this capacity depends on the column filling, its length and diameter, as well as on the anthocyanin source and concentration and has to be estimated for each new material. After cleaning, the materials were re-used five times, with no noticeable change in quality. With silica gel RP-C18, the flow was very slow even under vacuum. The applied particle size is unsuitable for bigger scales. In Amberlite XAD-7 filled columns, no problems were found concerning flow at all. Amberlite XAD-7 was successfully applied in a 36-litre-scale column with a similar elution profile but higher pigment concentrations in the main coloured fractions, which obviously increases with the larger scale.

Table 2 compares the purification effect and the anthocyanin concentration in the main coloured fractions after SPE of the most suitable materials in the 8 ml and 20 ml scale. Among all solid phases tested in this work, Amberlite XAD-7 turned out to be the favourite for technological purposes.

CONCLUSIONS

The investigations proved that SPE is a rather quick and highly efficient purification method for crude anthocyanin solutions. The resulting extracts are almost completely free of sugars, sugar alcohols, fruit acids and

TABLE 2
Sugar and colour content in crude juice and in the main coloured fractions after SPE

Solid phase	*Sugars* (*g litre*$^{-1}$)	*Sorbitol* (*g litre*$^{-1}$)	*Anthocyanins* (*g litre*$^{-1}$) *8 ml scale*	*Anthocyanins* (*g litre*$^{-1}$) *20 ml scale*	*Colour recovery* (%)
Crude juice	108·5	62·8	2·15	2·15	
RP silica gel 100, C-18	ND	ND	0·81	6·34	>97
Amberlite XAD-7	ND	ND	0·23	3·86	>95
Fractogel TSK CM 650 (H^+)	0·24	0·15	0·75		91

ND, not detected.

proteins, representing the base product for highly coloured anthocyanin concentrates or powders. Depending on anthocyanin charging and material chosen, the pigment is diluted or concentrated over the process. When ethanol is recycled, the anthocyanins are concentrated to a high extent. Acidification of eluent solvents was applied in order to inhibit colour degradation. In this preliminary study, two kinds of suitable adsorbent materials could be selected: ie reversed-phase silica gels and macroreticular non-ionic acrylic ester polymer resins, which we tested in 36-litre scale.

ACKNOWLEDGEMENTS

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ProductInformation

AMBERLITE XAD POLYMERIC RESINS
Sigma Prod. Nos. XAD-4, XAD-7, XAD-16, 1-0377,1-0393, and 1-0379

PHYSICAL DESCRIPTION:

Appearance: White translucent beads, sometimes with faint yellow cast

STORAGE / STABILITY AS SUPPLIED:

Amberlite[1] resins are stable for years stored at room temperature. They may be shipped as waterwet products with sodium chloride and sodium carbonate salts to retard bacterial growth. These salts must be washed from the adsorbent prior to use.

GENERAL REMARKS:

Three parameters affect binding capacity of a resin for a particular material. The dipole moment, the pore size and the surface area. The material to be adsorbed must be able to migrate through the pores to the adsorbing surface. For adsorbents of equal pore size, a larger surface area has higher capacity for solute. There is an inverse relationship between surface area and pore size: the smaller the pore size the greater the surface area. On the following page is a table of comparative physical data. More detailed information for each resin is available on request.

The nonpolar XAD resins are generally used for adsorption of organic substances from aqueous systems and polar solvents. For hydrophobic compounds up to MW 20,000, XAD-2 has been widely used, but it was discontinued by Rohm and Haas.[2]
Currently suggested:

1. for relatively low molecular weight (MW), XAD-4
2. for small to medium MW, XAD-16
3. for relatively large MW organics, X1180.

XAD-2 and XAD-16 have been used for removal of detergents from protein solutions. The capacity for XAD-2 for Triton X-100 was reported as 0.37 g per g dry resin (1.91 g of hydrated resin).[3,4] Most XAD resins are nonpolar and may be used over a pH range of 0-14, with maximum usage temperature 480°F.

XAD-7 is the only "moderately polar" XAD resin now available. It has been used to remove relatively polar compounds from non-aqueous solvents, and to remove non-aromatic compounds from polar solvents. It has been used for removal of organic pollutants form aqueous wastes, ground water and vapor streams. The pH range is 0-14, with maximum usage temperature 300°F. A similar product is Diaion HP2MG, having larger pore diameter, but very similar in surface area.

An extensive literature survey from 1987 to 1991 is available from Supelco as product number T412139. They are available from Supelco's Order Entry (800-247-6628-) or Technical Service (800-359-3041).

AMBERLITE XAD POLYMERIC RESINS
Sigma Prod. Nos. XAD-4, XAD-7, XAD-16, 1-0377,1-0393, and 1-0379

REGENERATING AND ELUTING AGENTS:

These resins are usually regenerated using methanol or other water-miscible organic solvents (acetone, isopropanol, etc.) If weak acids have been adsorbed, dilute base (0.1-0.5% NaOH) is effective; conversely, if bases have been absorbed, dilute acid (0.1-0.5% HCl) is suggested. Water can be used after adsorption is from an ionic solution. Hot water or steam is often helpful for volatile materials.[5]

Resins should be thoroughly washed before first use.

REFERENCES:

1. Amberlite is a trade name of Rohm and Haas Company.
2. XAD-2 is still available in research quantities from Supelco as # 2-2075 and #2-0279 (environmental testing grade).
3. *Anal. Biochem.,* 53, 304 (1973).
4. Supplier information for XAD-2.
5. Supplier information sheets.

RESIN	CAS NUMBER	CHEMICAL NATURE	DRY DENSITY (vs. wet) (g/mL)	SURF. AREA (sq. m/g)	PORE DIAM, AVE (Angstroms).	WEST MESH SIZE (nomimal)	PORE VOL. (mL/g)
XAD-2	9060-05-3	Hydrophobic polyaromatic (dipole moment 0.3) Used to remove hydrophobic compounds up to 20,000 MW	1.07 (1.02)	330	90	20 to 60	0.65
XAD-4	37380-42-0	Hydrophobic polyaromatic (dipole moment 0.3) Used to remove small hydrophobic compounds, surfactants; widely used in pharmaceutical manufacturing; used to remove chlorinated organics, pesticides, etc.	1.08 (1.02)	725	50	20 to 60	0.98
XAD-16	104219-63-8	hydrophobic polyaromatic (dipole moment 0.3) Used to remove hydrophobic compounds up to 40,000 MW; separation of large organic molecules, especially proteins. More efficient than XAD-2 (higher surface area).	1.08 (1.02)	900	100	20 to 60	1.82
XAD-1180 1-0377	9003-69-4	hydrophobic polyaromatic (less polar than XAD-4) Similar in application to XAD-4 in purification of enzymes, antibiotics, etc.	1.04 (N/A)	600	300	20 to 60	1.68
XAD-200 1-0393	N/A	hydrophobic polyaromatic Has been used in pharmaceutical manufacturing to adsorb steroids, amino acids, polypeptides, antibiotics from process streams	1.09 (N/A)	580	42	20 to 60	0.64
XAD-2010 1-0379	N/A	hydrophobic polyaromatic Formerly called AXT-204 Broad spectrum molecular weight range; used with biomolecules and pharmaceuticals	1.09 (N/A)	660	280	20 to 60	1.80
XAD-7	37380-43-1	Acrylic ester (dipole moment 1.8) Used to adsorb molecules up to MW 60,000; insulin recovery, metal ions, dry waste, organic removal and recovery; antibiotic recovery	1.24 (1.05)	450	90	20 to 60	1.14

1993

Chemically modified polymeric resins for solid-phase extraction and group separation prior to analysis by liquid or gas chromatography

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Order Number 9335017

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Chemically modified polymeric resins for solid-phase extraction and group separation prior to analysis by liquid or gas chromatography

by

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DOCTOR OF PHILOSOPHY

Department: Chemistry
Major: Analytical Chemistry

Approved:

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For the Major Department

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Iowa State University
Ames, Iowa

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CHAPTER 1. GENERAL INTRODUCTION

This research is presented in three parts. The first section deals with the use of derivatized resins for the extraction of phenols from aqueous samples. A resin modified by the addition of an acetyl group is impregnated in a membrane and analysis of several different water samples is performed.

In Part II a cation exchange resin is used to concentrate and fractionate bases and neutrals in aqueous samples. A multi-step elution process is used to fractionate neutrals, strong and weak bases. This same resin is also used to extract bases from nonpolar solutions.

Part III deals with the use of anion exchange resin for the concentration and group separation of acids and neutrals in aqueous solution. A variety of neutrals, phenols and carboxylic acids was studied. A two-step elution process was used for group separation. This resin was also used for the extraction of carboxylic acids from nonpolar solutions.

Polymeric Resins

The most common packing used today in Solid Phase Extraction (SPE) and High Performance Liquid Chromatography (HPLC) is silica gel. The gel is a network of silica atoms bound to one another by siloxane bonds.

This gel is then modified with any number of chemical entities such as C8 or C18 reacted at terminal hydroxyl groups to facilitate reverse-phase separations. Although these packings dominate the chromatography industry, as new and better polymers are being developed an increasing percentage of the market is turning to polymeric resins.

Although silica columns are very efficient, they do have some serious drawbacks. The most serious of these is their instability in alkaline or highly acidic solution. They also require an extended equilibrium time between runs when gradient elution in used. These packings possess residual silanol groups which cause peak broadening and tailing of polar compounds such as amines or alcohols.

Modern polymeric resins are unaffected by pH. In the past the big disadvantage of polymeric resins was a shrinking or swelling of the resin when subjected to solvent changes. Modern polymeric stationary phases are highly cross-linked, which gives them an increased stability towards changes in solvent strength. The most widely used polymeric resin is polystyrene divinylbenzene (PS/DVB). The chemical structure of this polymer is shown in Figure 1. Chemical derivatisation is easily achieved by Friedel-Crafts reaction on the benzene ring.

F. Nevejans et al. studied pore structure and its influence on polymeric stationary phase (7). They found that the microporosity was important to the chromatographic performance of the polystyrene. For hydrophobic solutes, the entire bulk of the resin entered into the adsorptive process, not only the surface. Solutes can penetrate the pores into the polystyrene matrix. The micropores vary with the nature of the solute and the eluent.

R. I. Greyson and A. M. Patch (8) compared PS/DVB and SiC_{18} for HPLC. They found that of the two reverse phase materials, the polymeric resin gave greater selectivity, higher capacity, but lower efficiency when compared to the silica resin.

A 1990 paper by Sun and Fritz (18) introduced a number of chemically modified polymeric resins for high-performance liquid chromatography. The resin was easily derivatized by a Friedel-Crafts reaction on the benzene ring of the polymer. These modifications included a hydrophilic acetyl group, a methyl cyano group, a tert-butyl group, and succinic acid group. Extensive studies were done in which the retention of various compounds was compared on the resins. The hydrophilic resins showed an increased retention of some polar compounds when compared to underivatized polymeric resins.

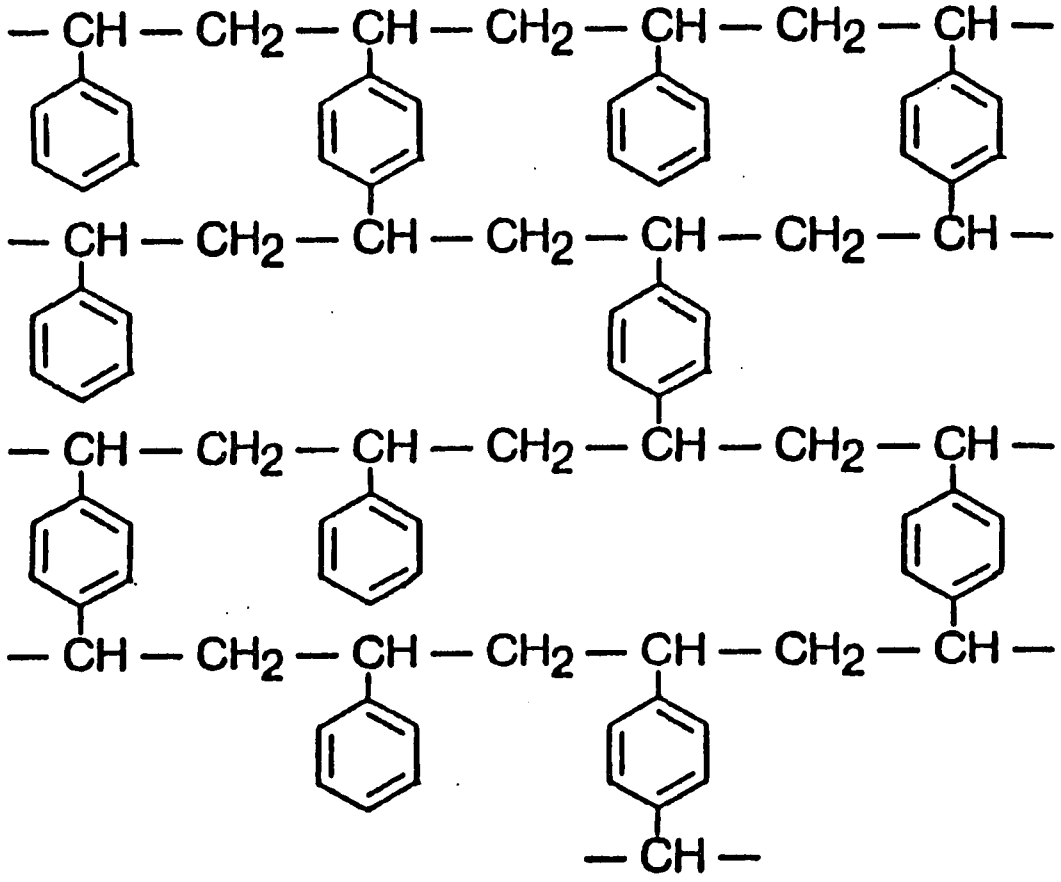


Figure 1. Chemical structure of polystyrene divinylbenzene. (PS/DVB).

Solid Phase Extraction

In most instances, "real world" (i.e. industrial by-product, environmental or biological samples, etc.) samples tend to be too dilute, too complex, or incompatible with chromatographic systems. This fact is often overlooked in analytical development, thus causing sample preparation techniques to be a limiting factor in many analyses. In comparison with other developments, it has been suggested that sample preparation advances have come about due more to need rather than desire (34). Regardless of original sample concentration and matrix, the ultimate goal of any sample preparation technique is a final solution with an enriched sub-fraction of analytes of interest at easily separated and detected concentrations that are compatibile with the chromatographic system of choice.

In a recent paper on organochlorine analysis (35), D. E. Wells and an IUPAC commission pointed out the major considerations in sample cleanup, extractions, and group separations. In trace analysis the concentration of the determinand is often close to the limit of detection (L.O.D.). Qualitative analysis requires a signal three times that of the baseline noise. The limits for quantitation are generally 5-10 times greater than the L.O.D. Background interference from the sample matrix, solvent, and induced

contamination adversely affect this signal/noise ratio. In order to minimize these influences, the general standards of good laboratory practice are essential to maintain sample integrity. Wells and his colleagues laid out the guidelines which a laboratory should follow regarding cleanliness, sample storage, and sample handling.

In the analysis of liquid samples, invariably the sample preparation step is the most time consuming step in the procedure. It is often the step in which the most analyte is lost and the majority of contaminants are introduced (via solvents, glassware, etc.). The standard E.P.A. methods for concentration and isolation of trace analytes in liquid samples call for liquid-liquid extraction. This technique is often considered to be a "mature" technology. But as pointed out in a review by Baird (9), many problems with the technique still exist. They include mass transfer of analyte in drop swarms, variation of drop size, formation of emulsions, effect of scale upon axial mixing, slow phase separation in extraction due to normally low differences in density between liquid phases and high viscosity, problems providing complete agitation of the sample to ensure intimate contact between the two phases, etc. These problems increase in importance as sample size increases. This paper illustrates the need for the development of better methods.

Liska and coworkers (1) have also pointed out other problems including the problems introduced when larger solvent volumes are used. Analytical chemists today realize that the large volumes of solvents generated in today's laboratories can add to environmental problems. Not only are solvents expensive to purchase, but their disposal is costly. In addition, trace contaminants present in the extracting solvent often interfere with the measurement of the analyte of interest.

In liquid/liquid extraction concentrations are performed by boiling or evaporating off the extracting solvent. This step can lead to significant loss of analyte, not to mention the hazards involved in heating toxic and flammable solvents. Trace contaminants in the organic solvent are also concentrated, causing them to become major interferences in the final chromatographic analysis. This step can also be very time consuming.

Extraction from liquid onto a solid adsorbent is a viable alternative to liquid/liquid extraction. Fritz and Junk (5) first described the use of polymeric resins for the extraction of phenols from aqueous solutions. A small column was filled with Rohm & Haas XAD-2 resin, and aqueous samples containing a variety of organic compounds were passed through these columns. These compounds were adsorbed onto the column

by hydrophobic attraction and subsequently washed from the column with a small amount of organic solvent.

In contrast, solid phase extraction offers a viable alternative to standard liquid/liquid extraction. SPE offers decreased solvent consumption, greater concentration factors, increased extraction efficiency, and is more easily automated.

An example of an SPE column is shown in Figure 2. Approximately 10 mg of resin is supported above and below by two PTFE frits. The column dimensions are 55 mm by 7 mm i.d. In Figure 3 we see a solution of the organic dye Arsenazo III passing through the resin. A tight band forms at the top of the resin as the complex is adsorbed on the resin by a hydrophobic interaction. By looking at the absence of color in the drop emerging from the column, we can see that the organic dye is completely taken up onto the resin. Figure 4 shows the elution of the dye in an acetone solution containing ammonia. By adsorbing from a large volume of aqueous solution and eluting with small volumes of organic solvents, significant concentration factors can be obtained.

As stated earlier, SPE is compatible with automated techniques. When compared to liquid/liquid extraction, which is often quite labor intensive with rather elaborate systems

Figure 2. Photograph of SPE column containing acetyl membrane plug

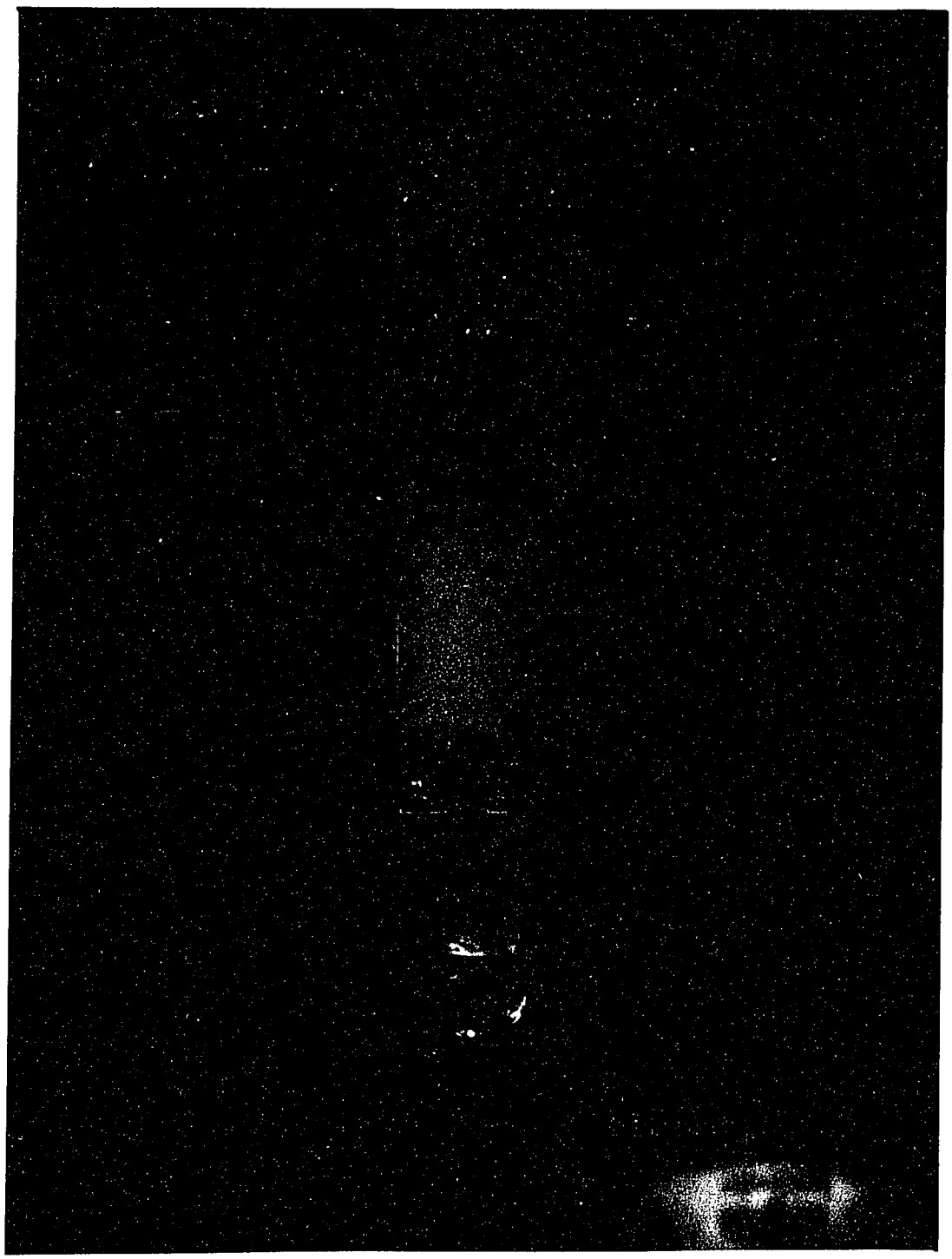


Figure 3. Photograph of Arsenazo III solution passing through SPE column, and extraction of colored complex.

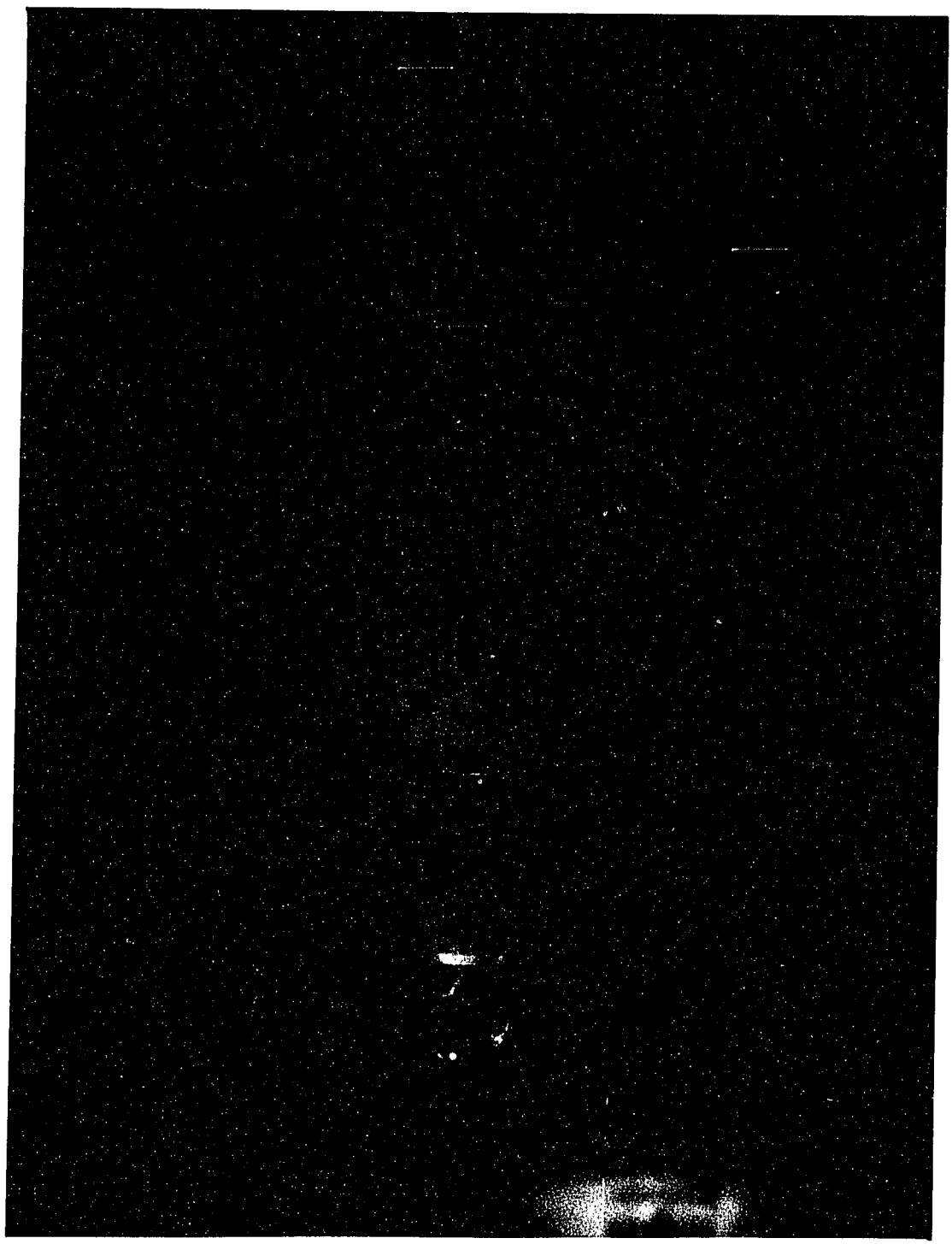
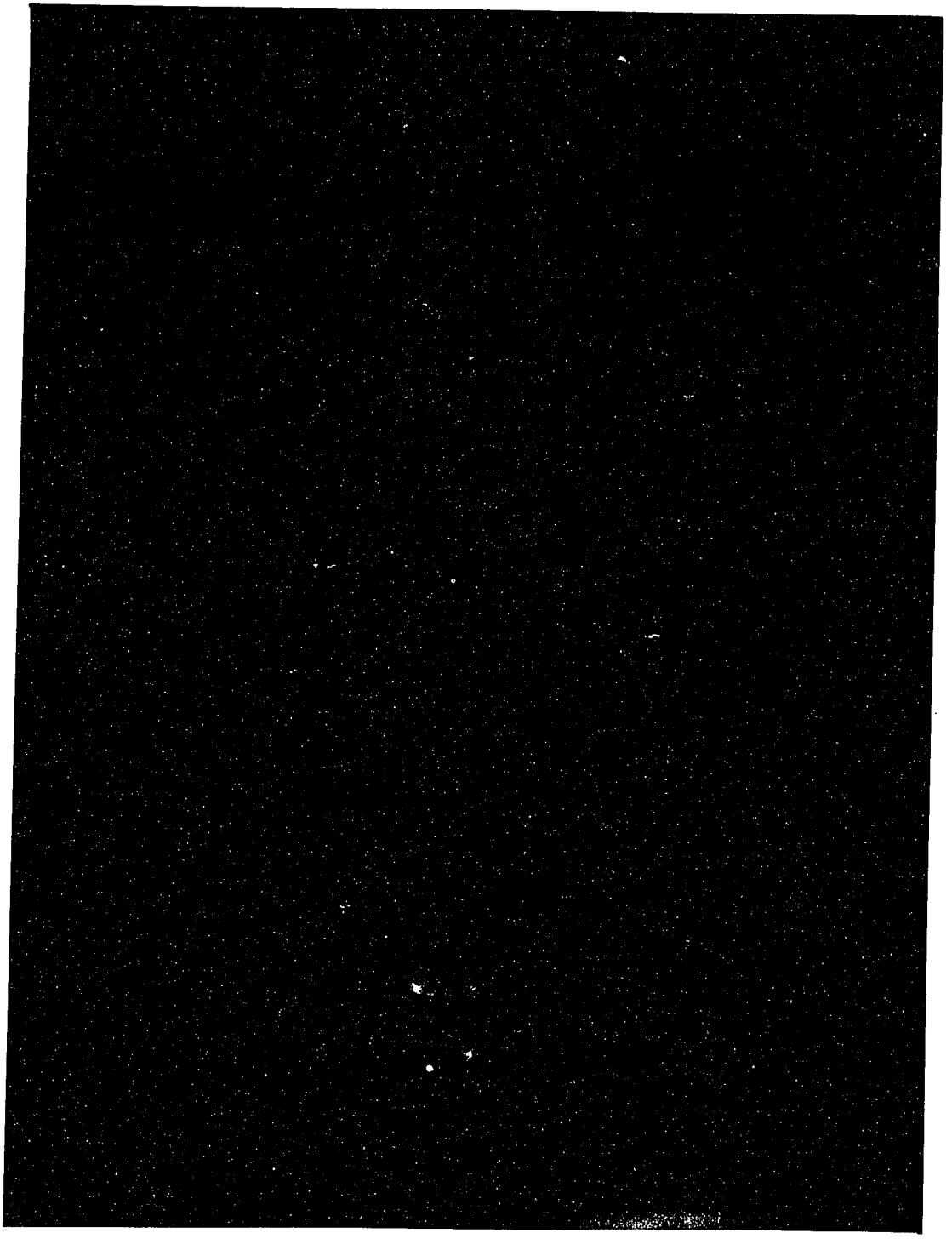


Figure 4. Elution step of solid phase extraction.



required for automation, SPE is amenable to on-line separations and concentrations.

M. Hennion described an on-line SPE concentration using a PRP-1 precolumn that could be switched into sequence with a C_{18} analytical column (13). The analytes were first concentrated out of a 10 ml aqueous sample, then the samples are eluted off the precolumn and separated on the second column with an acetonitrile/water eluent.

M. Chiba and others (16) constructed an automated SPE/HPLC system for the cleanup, preconcentration, and analysis of several pesticides. Eight pesticides were concentrated on a commercial C_{18} SPE column and eluted with acetonitrile. These concentrated pesticides were than analyzed by HPLC. Recoveries ranged from 84-93% with a standard deviation of less than 5%. Total analysis time was 90 minutes.

R. Majors and K. Fogelman (14) described a commercially available on-line SPE with a gas chromatograph. This instrument allows for an automatic cleanup and extraction with the SPE adsorbent of choice.

In 1991 J. Sun and J. S. Fritz (40) demonstrated a method for the on-line extraction of basic compounds by placing a sulfonated polymeric resin in the injection port of a gas chromatograph. This sulfonated resin, placed in the

injection liner and held in place with glass wool, extracted basic compounds from a sample solution while letting neutrals pass. The abstraction of basic compounds was essentially quantitative, but as yet no method has been developed for the elution of the adsorbed bases.

In similar work also by Fritz and Sun (42), a mercuric resin for the selective abstraction of mercaptans was developed. This resin was also placed in the split liner of a GC. It retained mercaptans, allowing other organic sulfur and non-sulfur compounds to pass.

There are many factors to consider when choosing a resin for SPE. G. Junk and J. J. Richard (17) pointed out the advantages of a smaller resin particle size in the concentration of pesticides and polycyclic aromatics. At this time (1988) polymeric resins were limited in diameter in comparison to silica based resins. Commercially available polymeric resins were much larger than their silica counterparts. They found that the smaller particle size of the silica resin made for a more efficient solid phase extractant. It facilitated higher flow rates and needed smaller volumes of organic solvent for complete elution. It was also more adaptable to hand-held syringes, making on site extractions possible. However, recent advances in the processes used in the preparation of polymeric resins by such

companies as Sarasep, Inc. (Santa Clara, CA), have made polymeric resins available in particle sizes comparable to those of porous silica.

Chiba and coworkers have studied the use of C_{18} silica columns for the automated extraction of pesticides from aqueous samples (45). They studied the effect on the extraction of the flow rate of sample through the sorbent bed, the amount of sorbent needed, and the sorbent packing particle diameter. For this application, they found a flow rate of 10 ml/min could be used, and the size and amount of resin varied dependent upon the desired application.

In another paper by G. Junk and coworkers (15), the interferences encountered when silica-based C_{18} bonded phase columns were used for SPE were studied. They found a large number of interferences in commercially available SPE columns. They found low level impurities could be extracted from the C_{18}-bonded porous silica, the polypropylene housing, and the polyethylene frits used to hold the resin in the tube. The intensity of the peaks is not only dependent upon the commercial column used, but also the eluting solvent used and its ability to extract low level impurities from polyethylene and polypropylene. A large number of silanol interferences were observed that could be attributed to hydrolysis of the C_{18}-bonded phase silica. It was suggested

that a solid phase more inert than porous silica would be useful.

SPE has demonstrated usefulness other than preconcentration and cleanup. Quite often isolation of a specific analyte can be achieved or, in other cases, fractionation of complex samples. M. Mills and E. Thurman at the University of Kansas used a mixed mode SPE scheme in the isolation of triazine metabolites from soil and aquifer sediments (44). The analytes were first extracted with methanol/water from the soil and then concentrated on a C_{18} column. They were then eluted with ethyl acetate. After this, the small volume eluate was passed through an anion exchange SPE column to remove coeluted humic substances. Recoveries from these quite difficult samples were ≈ 75%, with standard deviations of ≈15%.

The most widely used general technique used for the identification of volatile organic compounds in complex mixtures is GC/MS. In many cases, the gas chromatograph gives inefficient separation. In these instances, some type of group separation or fractionation is performed prior to analysis to simplify the mixture and gain some information about the chemical nature of the components. Liquid/liquid techniques are generally used in these instances (23,24,25). A 1981 paper by Svec and Colgrove (22) detailed a method for

fractionation of strong and weak acids, bases, polar and nonpolar compounds, aldehydes, and ketones by liquid/liquid extraction. This fractionation was achieved by performing multiple extractions from aqueous to methylene chloride with pH adjustments and Girard's Reagent-T for the ketones. While this was the most comprehensive study of liquid-liquid extraction for fractionation, the group separations reported were often incomplete, and recoveries ranged from 40-80% with an average of around 70% for most compounds. In contrast, the SPE method described here is faster, generates much less waste, and exhibits recoveries of 90% or better for compounds present at 10-40 ppm.

The EPA has directed an SPE method for the fractionation of nonpolar organic toxicants in aqueous samples using an methanol-water elution gradient (26).

In an EPA report put out in 1989 (27), V. Lopez-Avila and coworkers used a variety of SPE columns including fluorosil, silica gel, and alumina to fractionate a variety of PCB's and phenolic compounds.

F. Ulberth and E. Achs (28) used a commercially available silica-based column, CHROMABOND SA, to fractionate the methyl esters of a fatty acid mixture. Fractionation was achieved by varying solvent polarity. The recoveries were quite good (in most instances 95 - 100%).

N. Theobald (29) demonstrated the ability to fractionate petroleum hydrocarbons using reversed phase (C_{18}) and normal phase (SiO_2) SPE columns.

Aldridge and Oates (30) did similar work separating aliphatic and aromatic hydrocarbons using a C_{18} column. Although fractionation was not complete, it was adequate for the sample mixtures studied which included gasoline, diesel fuel, and samples taken from a suspected arson site.

Thompson and coworkers (36) performed a separation of basic and neutral drug fractions on a silica based copolymeric column.

J. R. Benson devised a scheme for the concentration of basic drugs on polymeric columns (37). Hexanesulfonic acid was used as an ion-pairing agent to extract amphetamine and methamphetamine from urine samples. Recoveries in both cases averaged >90% with a standard deviation of 6%.

Fritz and Kaczvinsky (38) demonstrated a method for the extraction of basic organic compounds from aqueous solution. The bases were taken up as cations on 2.5g of sulfonated polymeric resin. Recoveries were quite good, generally 90 - 100%, but large volumes of ammonia in methanol (40 ml) were needed for elution of the bases due to the large volume of resin needed. This large elution volume necessitated a concentration step prior to analysis.

Parry and coworkers at Supelco (39) used a weak cation exchange silica resin for solid phase extraction of four catecholamines from blood plasma. The amines were eluted from the resin $HClO_4$ in water and analyzed by HPLC. Recoveries of the basic drugs were all 90 - 100% with standard deviations of 5-10% for three trials.

In 1975, Fritz, Chang, and Chriswell first demonstrated the use of polymeric anion exchange resin for the solid phase extraction of phenols from water as phenolate anions (41). Sodium hydrosulfite was added to the solution in order to minimize the oxidation of phenol in basic solution. The concentrated phenols were eluted by first passing aqueous HCl through the resin to convert the phenols into their molecular form and then eluting with methylene chloride and acetone. Once again a rather large volume of eluent was needed, so a concentration step in which excess solvent was boiled off was performed. Recoveries of phenols from tap water were 90 - 100%.

A. DiCorcia and coworkers (31) have previously attempted a group separation of acids and neutrals. In this work a separation was performed using graphitized C black as an SPE adsorbent. Two modes of adsorption were achieved by utilizing naturally occurring positively charged centers for the electrostatic adsorption of anions, and hydrophobic

attraction for adsorption of neutrals. A rather large amount of adsorbent (250 mg) and eluent are needed, due to the fact that C black is a rather inefficient resin.

N. Yamaga and coworkers (32) have demonstrated a liquid-liquid method for the group separation of bile acids. They used a variety of internal standards added to the mixture in order to quantitate the acids present in each fraction.

J. Street and others (33) concentrated a mixture of bile acids on a C_{18} SPE column. They fractioned the samples into 3 groups: unconjugated, glycine- and taurine- conjugated bile acids by varying the polarity of the eluent (ethanol/ chloroform/ water mixture buffered with acetate).

SPE with Resin Impregnated Membranes

One of the biggest disadvantages of SPE is the formation of channels through the resin bed at large sample volumes. As more and more liquid is forced through the resin bed at relatively high pressure, the solution starts to form favored pathways, or "channels", through the column. This leads to decreased extraction efficiency due to a decrease in mass transfer efficiency and significantly lowers the effective capacity of the resin. The membrane technology, first introduced by Markell and Hagen of 3M in 1990 (10), tackles this problem. They embedded C_{18} resin particles in a polytetrafluoroethylene (PTFE) fibril network, thus holding

the resin firmly in place. The PTFE network consists of less than 10% of the total weight of the membrane, leaving 90% of the membrane available for adsorption sites. By impregnating the resin in a membrane, the individual resin particles are stabilized, thus inhibiting channel formation. This allows for higher flow rates and larger back pressures. In addition, it ensures that the entire resin bed will be put to use, thus maximizing the capacity of the resin.

This technology significantly decreases solvent consumption. To extract a pesticide analyte from 4 L of aqueous solution using liquid-liquid extraction, roughly comparable volumes of organic solvent must be used. In comparison, using a C18 empore disk for the extraction of pesticides from water (Barcelo and coworkers, ref. 11), a 4 liter sample containing 1 - 4000 μg of pesticide was concentrated on a extraction disk by passing the sample through the disk. The analyte was then eluted with 20 mL of methanol.

SPE disks have been used in the sample preparation steps in a number of methods for water analysis in environmental laboratories (19). These disks have shown significant improvements in methodology for water analysis. Higher throughput leads to shorter analysis time and larger concentration factors. The stabilized resin particles resist

the channeling effects suffered by loose resins at high flow rates and large volumes. Senseman, et al. (12) have demonstrated a significant advantage in pesticide stability when stored on extraction disks when compared to aqueous storage. A large variety of pesticides were studied with storage periods of up to 180 days and storage temperatures of -20°C to 4°C. They reported better recoveries of pesticides using the membrane for storage in all cases. Particularly unstable pesticides, such as Captan, showed markedly improved results when stored on the disk rather than in solution. It has also been suggested (19) that these disks be used for on-site sample collection, with the sample loaded disk being transported to the laboratory for analysis, rather than collecting large liquid samples and transporting these solutions to the laboratory.

While there are numerous examples of the use of SPE disks in the extraction of hydrophobic analytes, low recoveries are often obtained when more polar, water soluble analytes such as phenols are studied. Several techniques have been tried in an attempt to increase recoveries, including Ph adjustment to neutralize the phenol, addition of a salt to "salt out" the phenol, alternative sorbents, larger sorbent beds, ion pairing, and smaller sample volumes.

Sun and Fritz (6) have shown that resins made more hydrophilic by chemical derivatisation adsorb polar molecules more effectively than C18 particles. This modified resin allows for a more intimate contact to occur between the bulk solution and the resin surface.

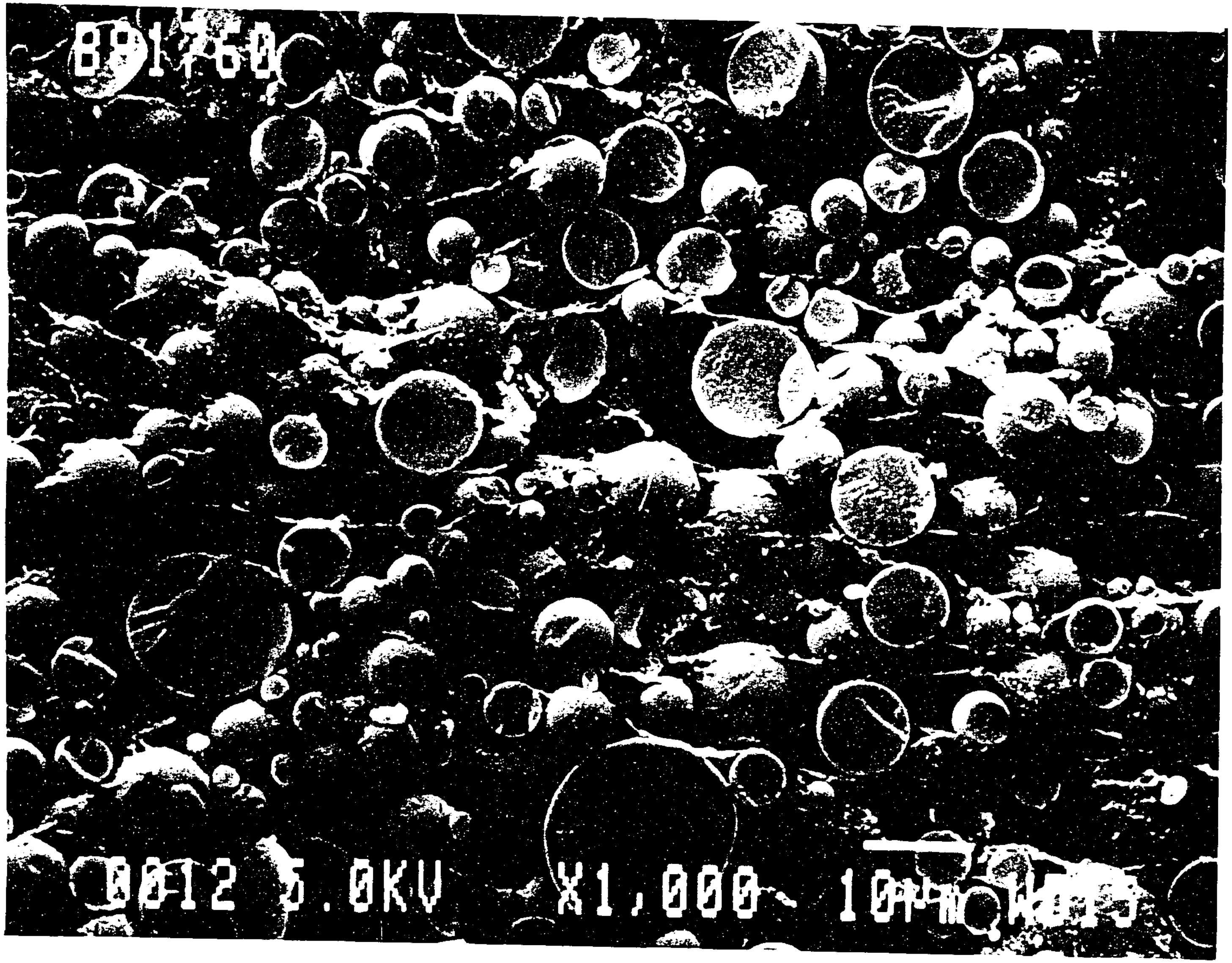
PART I. SOLID-PHASE EXTRACTION OF PHENOLS USING MEMBRANES LOADED WITH MODIFIED POLYMERIC RESINS.

INTRODUCTION

In 1989 a new family of materials for solid phase extraction (SPE) was introduced (10). This is a membrane, or disk of polytetrafluoroethylene (PTFE) fibrils impregnated with small 5-8 μm particles of adsorbing material such as C18 silica or poly-styrene divinyl benzene (PS/DVB). The mass of these membranes, developed and manufactured by the 3M company (St. Paul, MN, USA), is 90% resin particles, with the PTFE network being only 10%. This fibril network stabilizes the resin bed, prevents channeling, and improves mass transfer. An electron micrograph of such a membrane is shown in Figure 5. As illustrated, the bulk of the membrane is resin. Flow rates as fast as 200 ml/min are possible, yet uptake of organic solutes is efficient because of fast kinetics. These faster kinetics are a result of smaller particle size, close and uniform packing, and elimination of channeling when compared to standard loose packing SPE.

The discharge of phenolic compounds by industry and municipalities is of great concern to the public (20). There is great interest in the determination of phenols, as illustrated by the large amount of papers generated on the subject. The majority of the analyses, including the traditional E.P.A. method, call for liquid-liquid extraction. This is usually performed by acidifying the aqueous sample

Figure 5. Electron micrograph of acetyl membrane.



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containing the phenols and extracting with a roughly equal volume of methylene chloride. This is followed by a concentration step (boiling down) and then analysis by GC. Solid Phase Extraction is clearly a simpler and cleaner approach, and has been suggested by some (21). Traditional SPE supports (Si-C_{18}, PS/DVB, etc.), show good recoveries of some phenols, but are generally too hydrophobic to show good extraction efficiencies of phenol itself and others.

A 1990 paper by Sun and Fritz (18) introduced a number of chemically modified polymeric resins for high-performance liquid chromatography. Among these resins was an acetyl derivatized resin which displayed increased hydrophylicity. In a 1992 paper (6), Sun and Fritz demonstrated the usefulness of this resin for SPE. In comparison with silica C_{18} and underivatized resins, the acetyl resin was vastly superior in its ability to extract polar molecules from aqueous samples.

This work explores the use of resins impregnated with acetyl derivatized resins for the extraction of phenols from aqueous samples. The increased hydrophylicity of the resin encourages intimate surface contact between resin surface and solution and makes for a more efficient extraction of the more polar phenols.

The use of the membrane further improves the extraction. A novel method of incorporating these membranes into SPE tubes is also introduced.

EXPERIMENTAL

Apparatus

Solid-phase extractions were performed using the laboratory-made apparatus shown in Figure 6. It consisted of a 30 ml glass reservoir connected by a small adapter to the SPE column containing the membrane plug. The columns were connected to the reservoir by an adapter (Alltech Assoc., Deerfield, IL, U.S.A.). The SPE columns were obtained from Varian (Harbor City, CA, U.S.A.). The membrane was held in place above and below by polyethylene frits. The top of the reservoir has a ground glass joint which may be connected to a source of constant adjustable air pressure, which was used to control the flow rate of the liquid sample through the membrane.

A Hewlett Packard 5880 gas chromatograph with a flame ionization detector and an HP 5880A series 4 integrator was used to analyze all phenols. An HP 7673A autoinjector was used to introduce a 2 μl sample onto the column. Separations were performed using a fused silica capillary SPB-1 column (Supelco, Inc., Bellefonte, PA, U.S.A.).

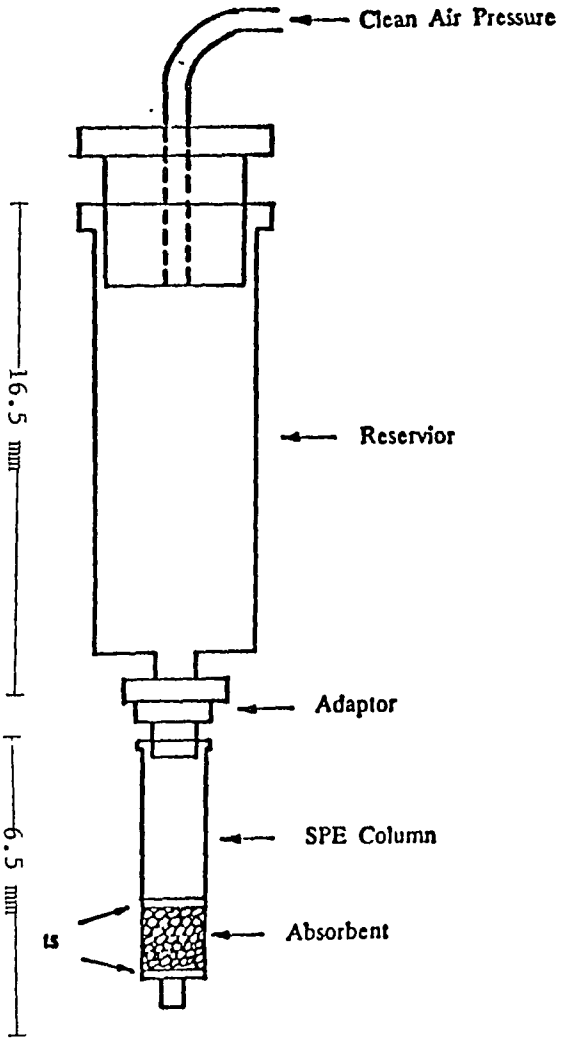


Figure 6. Laboratory made SPE apparatus.

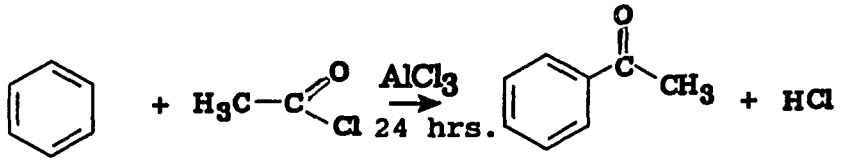
Reagents and Chemicals

The reagents and chemicals used for the derivatisation were reagent grade and were dried by molecular sieves. They were obtained from the Aldrich Chemical Company (Milwaukee, WI, U.S.A.). The phenols used in the experiment were analytical standards obtained from Supelco, Inc. (Bellefonte, PA). Laboratory distilled water was further deionized by a Barnstead Nanopure II system (Sybron Barnstead, Boston, MA, U.S.A).

Preparation of Modified Resins

The resin used in this experiment was a PS/DVB resin obtained from SRP 80 (Sarasep, Inc., Santa Clara, CA). The structure of this porous polymeric resin is shown in Figure 1 of the Introduction. It is spherical and has an average diameter of 8 μm and a surface area of about 400 m^2/g. The resin was rinsed with methanol, acetonitrile, and acetone before derivitisation.

The reaction used in the introduction of the acetyl functional group onto the resin was first described by Sun and Fritz (18) and is shown below:



The preparation consists of a simple one-step reaction. 5.1 g of resin was suspended in 30 ml of carbon disulfide. Anhydrous aluminum chloride (9.5 g) was then added as catalyst. To this mixture, 5.5 g of acetyl chloride was added dropwise. This mixture was lightly stirred and allowed to react at 50°C for 24 hours. The product was then poured into ice water to quench the reaction. This mixture was then filtered over a medium glass frit and rinsed with acetone, methanol, and water. The presence of the carbonyl group was confirmed by the presence of a strong FTIR band at 1690 cm^{-1}. A physical indication that the hydrophilic group has been introduced can be seen by adding a small amount of resin to water. The underivatized resin forms clumps and floats on the surface of the water, while the hydrophilic resin breaks apart easily and forms a suspension in the water. These reaction conditions give an acetyl capacity of 1.2 mmol/g as determined by oxygen analysis.

Procedure for SPE

Membranes containing the acetyl resin were prepared by the 3M company (St. Paul, MN, U.S.A.) using resin prepared here at Iowa State University. Two types were prepared, one approximately 1 mm thick, and another approximately 3 mm thick. In this experiment only the thicker membrane was tested.

A membrane circle with a diameter of about 7.5 mm was cut out of the membrane sheet. This plug was then forced into the SPE column (7 mm i.d. by 55 mm) with 20 μm polyethylene frits above and below for support. Physical pressure was then applied to the membrane, causing it to form a seal with the edges of the SPE tube. This column was then attached to the SPE apparatus shown in Figure 6.

The sample solution was prepared by adding a standard solution of phenols (50 ppm each) in methanol to 20 to 30 ml of water solution so that the final concentration was approximately 0.4 ppm each. The Ph of the solution was lowered to approximately 2 with sulfuric acid to repress ionization of the more acidic phenols.

Prior to each use a small amount of methanol (ca. 1 ml) was added to the column to "prewet" the membrane. Without allowing the membrane to dry, the sample solution was passed through at a flow rate of 2 ml/min by applying a pressure of 15 p.s.i. The column was then rinsed with 2 ml of distilled water.

The phenols were then eluted from the column with 0.75 ml of either methanol or methylene chloride. This gives a concentration factor of approx. 25 to 40. Either eluant was found to be sufficient to completely wash off the extracted phenols. The eluate was collected in a 1.8 ml GC vial, to

which 0.1 ml of internal standard (500 ppm quinoxaline) was added. The vial was then capped and mixed with an orbital stirrer.

A 2 μl aliquot of the sample mixture was then injected into the gas chromatograph with a split ratio of 1:40. Helium carrier gas was used at a flow rate of 15 ml/min. The oven temperature was held initially at 65 °C for 2 minutes, and then ramped at 15°C/min to a final temperature of 250 °C. Detection was by flame ionization detector. Identification of phenols was based on retention time comparison of sample with standard. The recoveries were calculated by comparison of relative peak height to that of a standard consisting of the original methanol solution with internal standard added not subjected to SPE. To obtain an average recovery, a minimum of 3 trials was used in all cases.

RESULTS AND DISCUSSION

The most common mode of SPE in chemical analysis is with small tubes or cartridges filled with loose polymeric resin or bonded-phase silica particles. Usually a bed height of > 1 cm is used to ensure good retention of the desired sample compounds. However, this necessitates a relatively large volume of solvent (>1 ml) to quantitatively elute the adsorbed compounds. By packing the tubes with membrane disks 5-7 mm in diameter, more efficient elution is possible due to the shorter resin bed height. It was found that elution volumes of 0.25 ml were sufficient to elute the phenols quantitatively. The reason for this is that in the membrane the resin particles are smaller (5-8 μm vs.40 μm), more closely packed, and more evenly dispersed throughout the membrane when compared to loose packed resins. Since the particles are immobilized, it is possible to avoid the channelling that would be likely in a tube containing a shorter loose resin bed.

Earlier work done by Dr. Jeff Sun (Iowa State University, unpublished) had focussed on trying to incorporate these membranes into SPE tubes to take advantage of the physical stability of the resin when embedded in the membrane. Six of the normal 1 mm thick membranes were stacked within the SPE tube, and held in place with frits.

Results were good for some runs, but erratic. Tests with an azo dye, pyridylazonaphthol, showed that channeling occurred on the outer rim of the disks. It took great care to ensure that the disks fit tightly in the SPE tube.

It appeared that a single, thicker membrane would work well for SPE in a small column. A membrane of thickness of about 3.5 mm was prepared for us by the 3M company. A plug slightly larger (7 mm diameter vs. 6.5 mm) than the diameter of the SPE tube was then cut out of the membrane sheet with a cork borer. This plug is shown in Figure 7. The plug was gently forced into the SPE tube with PTFE frits above and below for support. The excess membrane served to form a tight seal with the edge of the tube. Tests with an azo dye showed no channeling around the outer edges, thus solving the problems seen with the thinner membranes.

Deionized Water

The first tests were run on 30 Ml deionized water samples spiked with phenol standards and acidified with H_2SO_4. The samples were passed through the acetyl membrane in the SPE column at a flow rate of 1 mL/min. The phenols were adsorbed onto the resin by hydrophobic interactions and subsequently eluted with methanol or methylene chloride. Tests showed an elution volume of 0.25 mL of either solvent was adequate to elute >95% of the phenols. In the GC step of

Figure 7. Photograph of acetyl membrane plug.

the analysis, however, a volume of ≈ 0.75 mL was needed in the vial in order for the needle in the autoinjector to reach the solution, so elutions were performed using 0.75 ml of methanol. An example of the chromatogram obtained is shown in Figure 8. The average recoveries (n = 3) of a large number of phenols is given in Table I. Recoveries of the phenols were quite good, with most recoveries in the 95 to 100% range.

Tap and River Waters

Realizing that the deionized water samples may be more ideal and not representative of the types of samples found in the real world, similar experiments were run on spiked tap and river water samples. Samples of both types were spiked to phenol concentrations of 0.4 ppm and 20 ml of these solutions were acidified and passed through the membrane. Once again the phenols were eluted with methylene chloride and analyzed by GC. An example of the chromatogram obtained is shown in Figure 9. Recoveries of the phenols from these real world samples were also quite good, with the exception of 2-methyl-4,6-dinitrophenol in tap water, the recoveries were generally >90%. The recoveries are reported in Tables II and III.

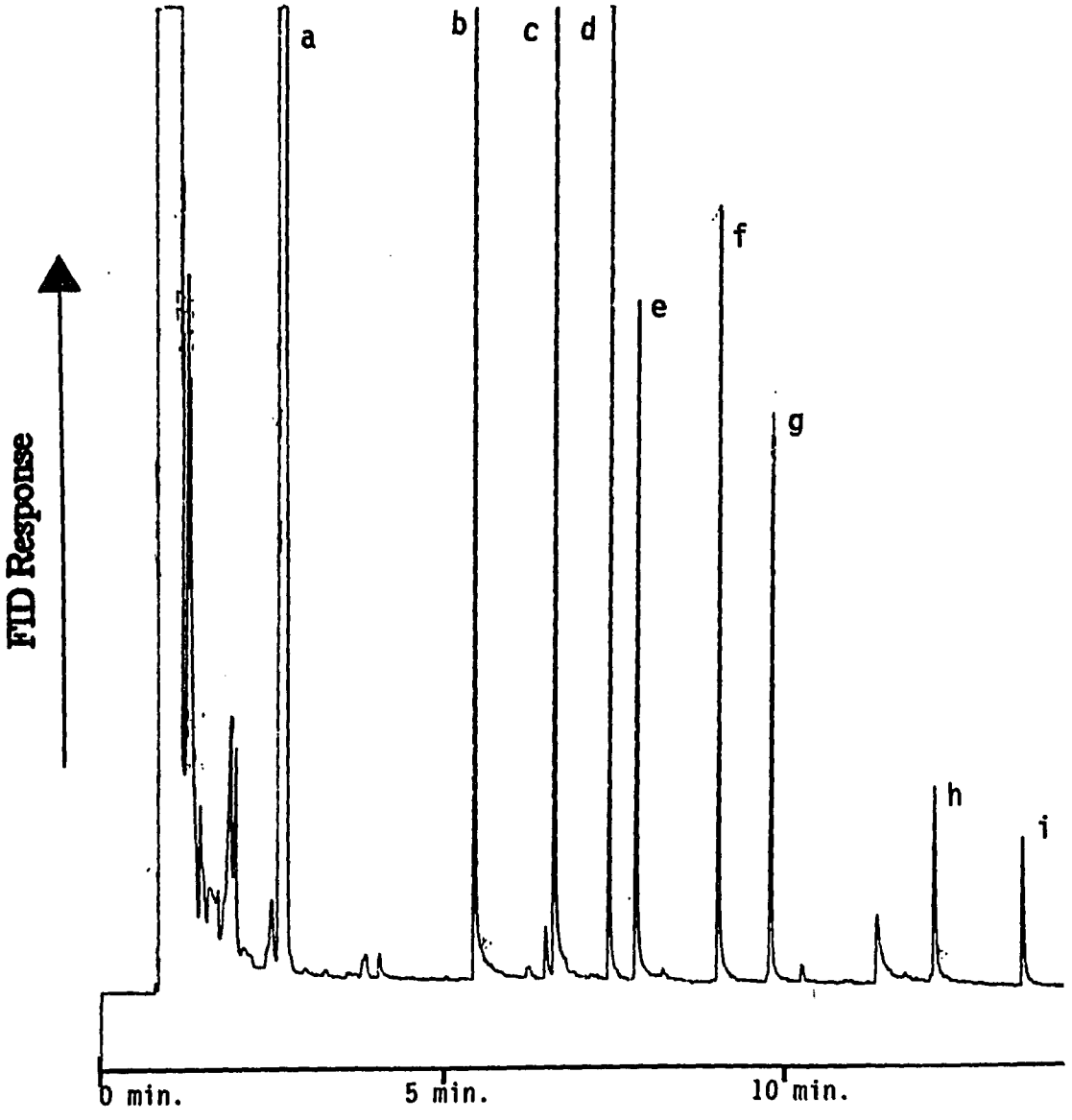


Figure 8. Gas chromatogram of phenols concentrated from deionized water solution.

Table I. Percent recoveries of phenols from deionized water on acetyl membrane. Elution with 0.75 mL of methanol. (n = 3)

Analyte	% Recovery	Std. Dev.
Phenol	99	2
3-Methylphenol	95	1
2-Nitrophenol	100	4
2,4-Dichlorophenol	98	0
4-Chloro-3-methyl phenol	98	0
2,4,6-Trichlorophenol	102	0.5
Pentachlorophenol	100	2
2-Chlorophenol	99	2
2-Methylphenol	99	4
4-Methylphenol	97	5
2,4-Dimethylphenol	97	5
2,6-Dichlorophenol	100	5
2,4,5-Trichlorophenol	96	5
2,3,4,6-Tetrachlorophenol	99	3
2-sec-Butyl-4,6-dinitrophenol	101	4
2-Methyl-4,6-dinitrophenol	94	2

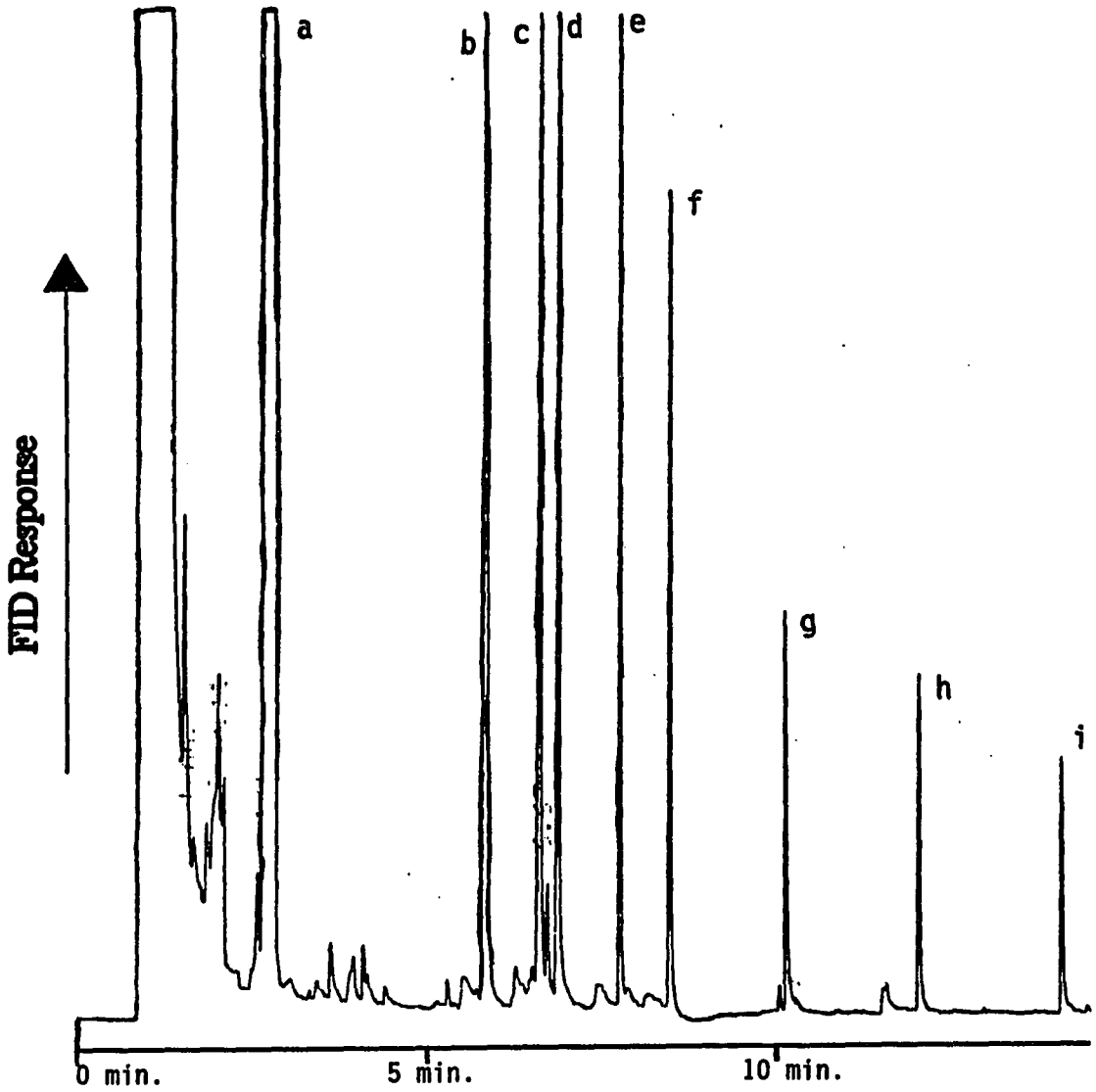


Figure 9. Gas Chromatogram of phenols extracted from Skunk River water and eluted with methylene chloride. {a=toluene (int.std.),b=2-chlorophenol,c=2-methyl phenol, d=4-methylphenol,e=2,4-dimethyl phenol, f=2,6-dichlorophenol, g=2,4,5-trichlorophenol, h=2,3,4,6-tetrachlorophenol, i=2-sec-butyl-4,6-dinitrophenol}.

Table II. Percent recoveries of phenols from tap water using acetyl membrane.

Analyte	% Recovery	Std. Dev.
Phenol	96	2
3-Methylphenol	93	2
2-Nitrophenol	102	3
2,4-Dichlorophenol	97	3
4-Chloro-3-methyl phenol	85	2
2,4,6-Trichlorophenol	98	3
Pentachlorophenol	92	1
2-Chlorophenol	106	4
2-Methylphenol	100	3
4-Methylphenol	98	2
2,4-Dimethylphenol	94	2
2,6-Dichlorophenol	108	8
2,4,5-Trichlorophenol	95	5
2,3,4,6-Tetrachlorophenol	95	9
2-sec-Butyl-4,6-dinitrophenol	97	4
2-Methyl-4,6-dinitrophenol	75	4

Table III. Percent recoveries of phenols from Skunk River water on acetyl membrane. (n = 3)

Analyte	% Recovery	Std. Dev.
Phenol	97	3
3-Methylphenol	92	4
2-Nitrophenol	98	1
2,4-Dichlorophenol	97	2
4-Chloro-3-methyl phenol	93	4
2,4,6-Trichlorophenol	101	0
Pentachlorophenol	100	4
2-Chlorophenol	103	2
2-Methylphenol	100	1
4-Methylphenol	98	4
2,4-Dimethylphenol	103	5
2,6-Dichlorophenol	104	6
2,4,5-Trichlorophenol	107	3
2,3,4,6-Tetrachlorophenol	101	0.5
2-sec-Butyl-4,6-dinitrophenol	101	2
2-Methyl-4,6-dinitrophenol	95	2

CONCLUSION

Phenols in the low ppm concentration can be effectively concentrated from aqueous samples by SPE with a membrane loaded with acetyl PS/DVB resin particles. Larger sample volumes can be passed through these membranes with no obvious channeling effects. Very small volumes of organic solvent are needed to elute the adsorbed phenols, leading to very large concentration factors. This technique appears to be superior to other standard methods of phenol concentration and cleanup, as it is rugged, quick, and efficient.

PART II. ION EXCHANGE PRECONCENTRATION AND GROUP SEPARATION OF BASIC AND NEUTRAL ORGANIC COMPOUNDS

INTRODUCTION

Solid-Phase Extraction (SPE) is fast becoming the preferred technique for analytical preconcentration (1). It uses much smaller amounts of organic solvents than liquid-liquid extraction. SPE is easily automated and is capable of obtaining very high concentration factors.

SPE is usually carried out with a very small tube (or column) packed with a spherical solid of small particle size and high surface area. It can be thought of as low performance liquid chromatography. Maximum retention is desired for the substances of interest with minimum retention of the other sample materials. The adsorbed substance can be subsequently eluted by a very small volume of an organic solvent or other suitable solvent.

The use of a short ion-exchange precolumn is well established (38,39) to concentrate anions or cations prior to their separation and determination by ion chromatography. In the present work an ion-exchange resin with suitable properties is used to preconcentrate organic substances from aqueous samples and then separate them into two fractions: neutral and basic compounds. The key to this separation is adsorption in a dual mode: neutrals by a hydrophobic attractions at unreacted sites on the sulfonated resin, and

protonated bases by electrostatic attraction at ion exchange sites. Figure 10 shows an illustration of this dual mode adsorption. The basis for this separation then is that neutral organic compounds are readily eluted from the SPE column by an organic solvent while basic compounds remain on the ion exchange resin as protonated cations. The latter can then be eluted by an organic solvent containing a base to neutralize the protonated solute cations. An outline for this 2-step elution is shown in Figure 11. This same principle has been used previously (2,3,4), but not to any extent for simultaneous preconcentration and group separation.

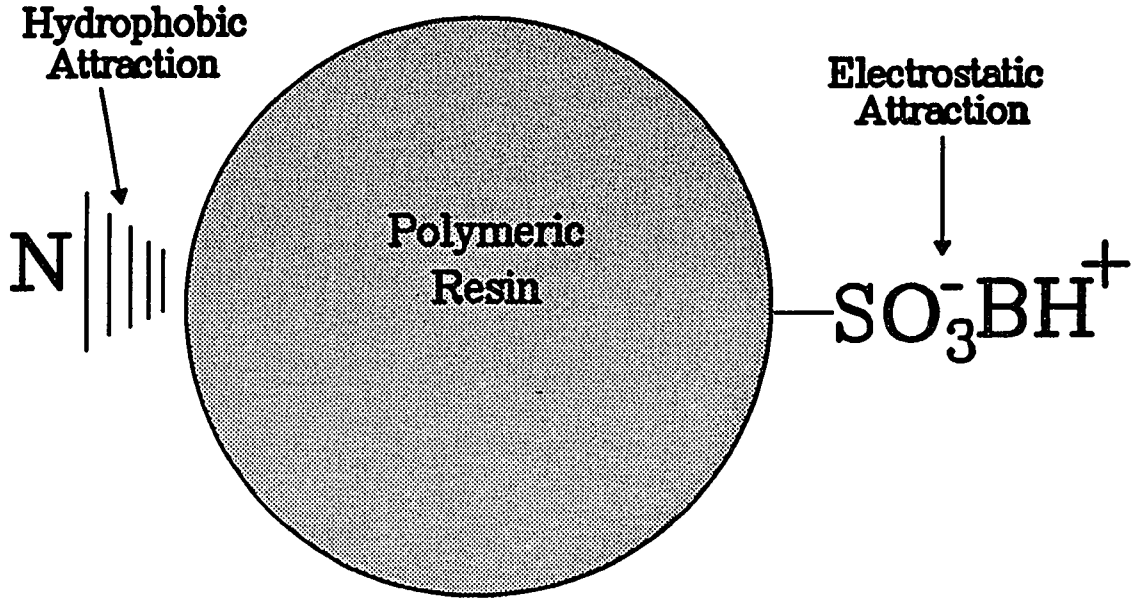
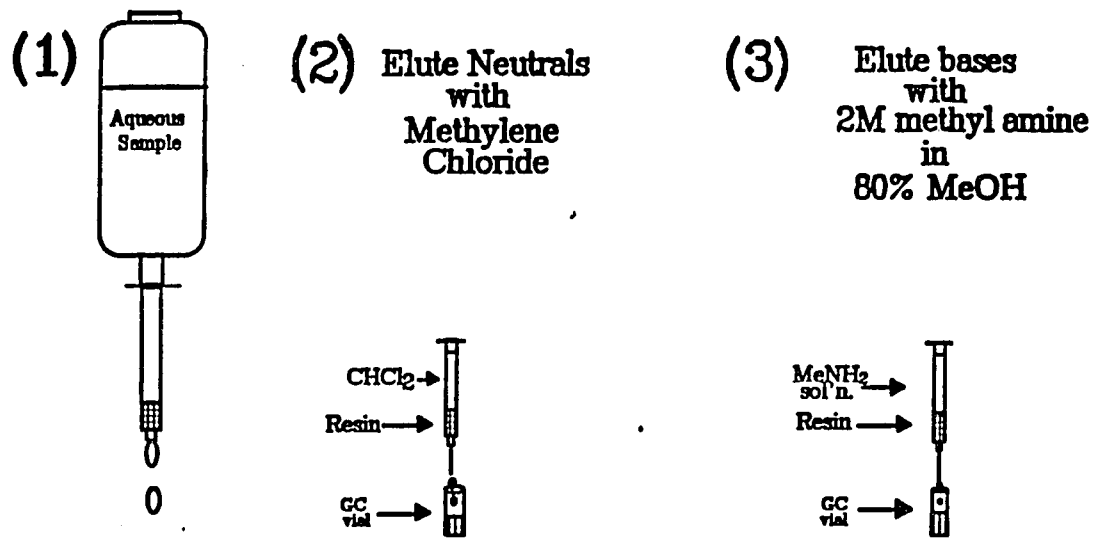


Figure 10. Diagram illustrating dual mode adsorption at single polymeric resin particle.



Experimental Procedure for preconcentration using cation exchange resin

1. The pH of the aqueous solution containing basic and neutral compouds was lowered with HCl.
2. The column containing the cation exchange resin was pretreated with 2 mL concentrated HCl, rinsed with 25 mL D.I. H_2O, and then wetted methanol,
3. The aqueous sample solution is then passed through the SPE column at a flow rate of about 1 mL/min.
4. The neutrals are eluted first using $CHCL_2$.
5. The bases are then eluted using Methyl amine or ammonia in methanol

Figure 11. Scheme for the group separation of acids and neutrals using anion exchange resin and 2 step elution process.

EXPERIMENTAL

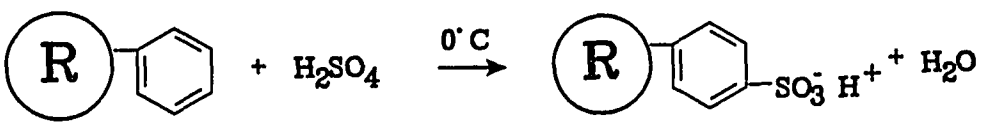
Reagents and Chemicals

The reagents used for the derivitisation of the ion exchange resins were of analytical grade. Reagents used and analytes studied in the solid phase extraction were >99% pure and used as obtained from Aldrich, Fisher, and Eastman-Kodak. Laboratory distilled water was further purified using a Barnstead Nanopure II System (Sybron Barnstead, Boston, MA, USA).

The resins were prepared using a highly porous, cross-linked polystyrene resin, Amberchrome 161 (Supelco, Bellefonte PA, USA). This spherical resin has an average particle size of about 40 μm and a surface area of about 720 m^2/g. An 8 μm PS/DVB resin, SRP-80 (Sarasep, Inc., Santa Clara, CA), was used in the membranes for the separations of bases and neutrals in nonpolar solutions.

Preparation of Cation Exchange Resin

A simple one step reaction was used for the preparation of the cation exchange resin. The time of the sulfonation reaction was the parameter adjusted in order to control the extent of the reaction. The reaction was carried out at 0 °C and is shown below:



PS/DVB

The procedure for the preparation of the sulfonated cation exchange resin follows. In an ice bath, 1 gram of resin was wetted with 1 mL of glacial acetic acid and stirred to form a slurry. To this slurry 25 ml of concentrated sulfuric acid was added. In the case of the Amberchrome resin, the reaction was stopped after 90 seconds by adding an excess of ice water. The reaction was allowed to proceed for 6 minutes in the case of the Sarasep resin. The final product was then rinsed with methanol, water, and acetone. The cation exchange capacity was then determined by first passing 5 ml of 1M HCl through a known mass of resin to assure the resin was all in the protonated form. The resin was then rinsed with deionized water and tested with litmus paper to assure that any excess acid was washed from the reservoir. Then 5 ml of a standardized NaOH solution was passed through the column. This was followed by another rinse with deionized water. The NaOH solution and water rinse was collected and titrated with a standard HCl solution. The capacity of the sulfonated Amberchrome resin was determined to be approx. 1.1 meq/g, while the Sarasep resin was approx. 0.6 meq/g.

The apparatus used for the solid-phase extraction (SPE) is shown in Figure 6 in the previous section. The SPE columns were obtained from Varian (Harbor City, CA, USA).

Approximately 100 mg of the resin was packed into the 55 x 6.5 mm I.D. columns. The resin was held in place above and below by 20 μm polyethylene frits. The resin bed height was approximately 12 - 15 mm. The columns were connected to the laboratory-made reservoir by an adapter (Alltech Associates, Deerfield, Ill, USA). The flow rate was controlled by the air pressure applied to the reservoir.

The concentrated samples collected were analyzed using an HP 5880A gas chromatograph with a flame ionization detector, an HP 5880 A Series Level 4 integrator, and an HP 7637A automatic sample injector (Hewlett-Packard, Avondale, PA, USA). The capillary column used was a Supelco (Bellfonte, PA, USA) SPB-1.

Procedure for SPE

Prior to initial use, the sulfonated columns were cleaned by passing about 2 ml of methanol and acetonitrile through them. This was followed by approximately 5 ml of a 2 M solution of HCl in methanol in order to protonate the sulfonic acid groups on the resin. The columns were then "wetted" with approximately 1 mL of methanol. The resin was not allowed to dry before passing the sample solution through the column.

The sample solutions were then prepared by adding a dilute methanol solution of several basic and neutral

compounds to 10 ml of deionized water so that the final concentration of each compound was 5 ppm. In the base-neutral group separation, the pH of this solution was then lowered to ≈ 2 with HCl. In the separation of strong bases from weak bases and neutrals a 0.1 M buffer solution of sodium phosphate was used to adjust the pH to 7. The sample was then added to the reservoir and the air pressure adjusted to give a sample flow rate of approximately 1 ml/min. The column and reservoir were then rinsed with about 5 mL of water and air dried for several seconds.

The column was then rinsed with 1 ml of methylene chloride to elute the neutral fraction. This was collected in a GC vial. An internal standard of 0.1 ml of 500 ppm quinoxaline in methanol was then added to the vial. The vial was capped, mixed with an orbital stirrer, and then analyzed by GC. A 1 μl aliquot was injected with a split ratio of 1:40. Helium carrier gas was used at a flow rate of 15 ml/min. The oven temperature was held initially at 65 °C for two minutes, then ramped at 15 °C/min. to a final temperature of 225 °C. The basic fraction was then eluted with 1 ml of either 2 M methylamine or 2 M NH_3 in methanol. Quinoxaline was also added as an internal standard as before. The vial was then capped, mixed, and analyzed by GC under the

previously given conditions. Recoveries were calculated by comparing the relative peak heights of collected samples to those not subjected to SPE. All results are an average of multiple trials ($n \geq 3$).

RESULTS AND DISCUSSION

Group Separation of Neutral and Basic Compounds

Previous work has shown that polystyrene divinyl benzene (PS/DVB) beads of high surface area (typically ≈ 400 to 750 m^2/g) are very efficient for SPE of low concentrations of organic solutes in aqueous samples (5,6). These resin beads still retain organic solutes when the beads are sulfonated provided that the degree of sulfonation is rather low. The sulfonated beads are able to retain protonated amine cations by an ion exchange mechanism. So long as the amines are present as cations, they are not washed off the resin by an organic solvent.

An intermediate capacity which facilitates adsorption by both ion exchange and hydrophobic interactions was sought. An optimum capacity of 0.6 to 1.0 meq/g was found. Lower capacities not only limited the amount of bases that could be adsorbed, but also showed less retention of neutrals. This could be largely due to the added hydrophilicity the exchange sites offered, causing more interaction between resin surface and solution. Larger capacities gave poorer retention of neutrals due to the reduction of underivatized surface area. The extent of sulfonation, as indicated by the exchange capacity of the sulfonated resin, can be kept low by sulfonation at 0°C for only a short period of time.

The scheme for preconcentration of small amounts of organic solutes and their separation into groups was as follows. The sample was adjusted to an acidic pH with hydrochloric acid. This converted the basic compounds to the protonated cations. Then the sample was passed through a small column containing sulfonated resin in the H^+ form at a flow rate of approximately 1 ml/min. The neutral organic solutes were eluted as a group with a small volume of methylene chloride. Then the basic fraction was eluted with a small volume of methylamine or ammonia in methanol.

Table IV shows excellent recovery of neutral organic solutes in water at 5 ppm. The gas chromatogram obtained is shown in Figure 12. In each case essentially complete elution is obtained with methylene chloride. The recoveries of some basic organic solutes studied are summarized in Table V. None of these compounds was eluted with methylene chloride except 2,3-dimethyl quinoxaline which is an extremely weak base and therefore behaves almost like a neutral compound. Some of the basic compounds were eluted well with methanol containing aqueous ammonia (see Figure 13), but stronger bases like hexylamine and cyclohexylamine were not eluted. However, as shown in Figure 14, methylamine in methanol eluted all of the bases efficiently.

Table IV. Percent recoveries of neutral organics on sulfonated resin.

Compound	% Recovery
4-Nitroacetophenone	98
Nitrobenzene	99
Benzothiazole	92
Octyl alcohol	94
o-Hydroxyacetophenone	99
Benzonitrile	95
Toluene	98
trans-2-Hexenylacetate	96
Butyl benzene	95
Anisole	90
Octyl aldehyde	97
Ethyl crotonate	88
Propyl benzene	94
1-Octanol	94
Benzonitrile	95

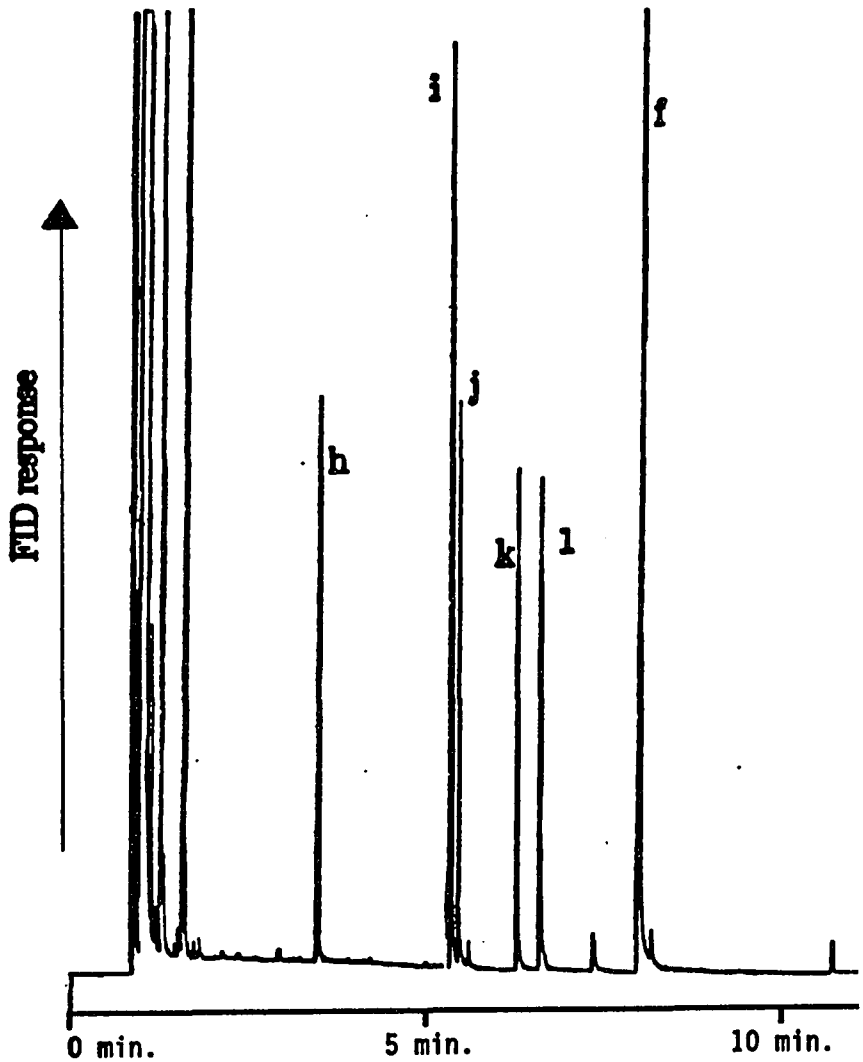


Figure 12. Gas chromatogram of neutral fraction concentrated on sulfonated resin and eluted with methylene chloride. {h=ethyl crotonate, i=propyl benzene, j=1-octanol, k=benzonitrile, l=nitrobenzene, and f=quinoxaline (int. std.)}.

Table V. Percent recoveries of bases concentrated on sulfonated resin.

Compound	% Recovery (NH_3)	% Recovery ($MeNH_2$)
Pyridine	91	91
Aniline	95	99
N,N'-Dimethyl aniline	61	92
Quinoline	92	96
Butyl amine	0	103
Octyl amine	0	97
Quinaldine	92	95
2,3-Dimethyl quinoxaline	63	63
Hexyl amine	0	95
Cyclohexyl amine	0	98
N-Methyl aniline	-	92
sym-Collidin	-	95
Phenethyl amine	-	98
Ethyl pyridine	-	93
Isopropyl pyridine	-	101
2,4-Lutidine	-	101

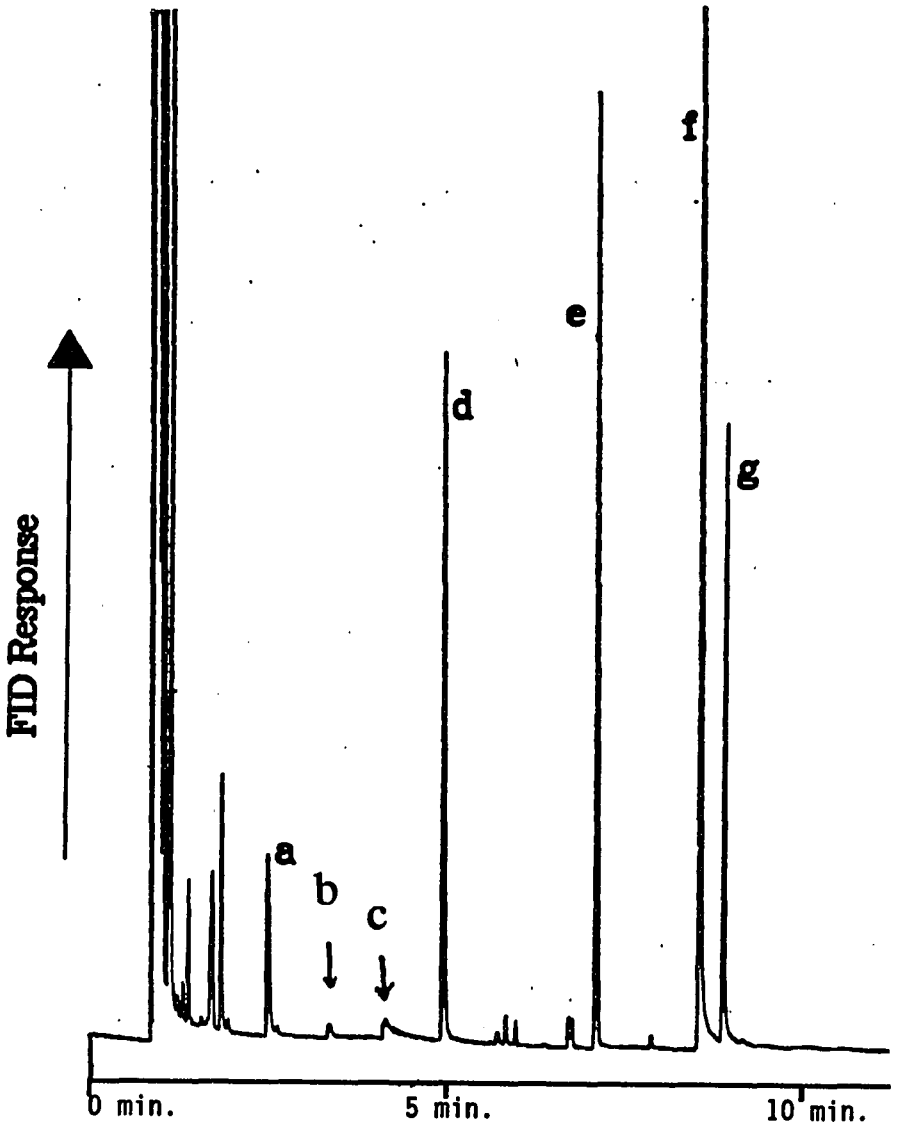


Figure 13. Gas chromatogram of basic fraction concentrated on cation exchange resin and eluted with ammonia in methanol. (a=pyridine, b=hexyl amine, c=cyclohexyl amine, d=2,4-lutidine, e=N,N'-dimethyl aniline, f=quinoxaline (int. std.), g=quinoline). Note absence of peaks for strong bases.

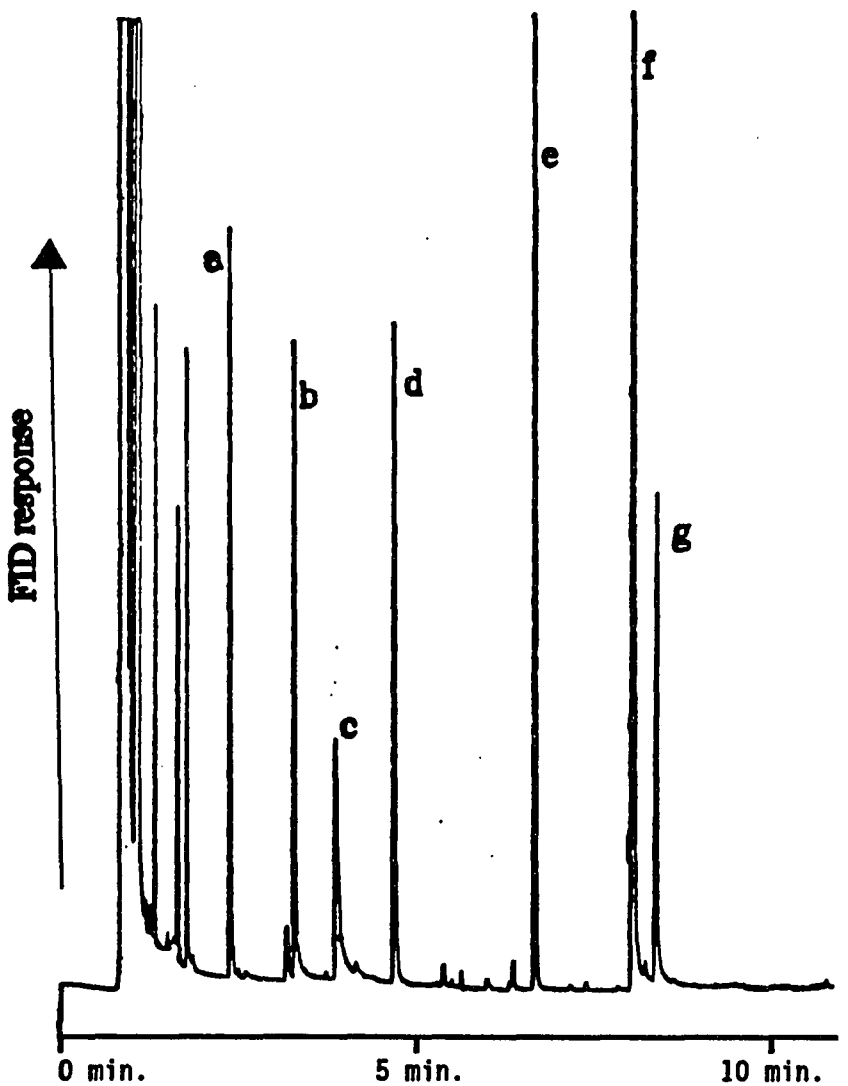


Figure 14. Gas chromatogram of basic fraction concentrated on cation exchange resin and eluted with methylamine in methanol. (a=pyridine, b=hexyl amine, c= cyclohexyl amine, d=2,4-lutidine, e=N,N'-dimethyl aniline, f=quinoxaline (int. std.), g=quinoline).

Methylamine (pK_b in water = 3.1) is a stronger base than ammonia (pK_b= 4.8). In the GC step methylamine is quite volatile and elutes well before any of the sample solutes. This property of ammonia allows a three-step elution process that further fractionates the bases. First, methylene chloride is added to elute neutrals. The second elution is then ammonia in methanol or acetonitrile. In this step the weaker bases (pK_b>5) such as quinoline or quinaldine are eluted. Then in the final elution, methylamine in acetonitrile is used as the eluent. This will then elute the remaining stronger amines.

The effect of pH on the group separation was investigated. Results were compared for samples buffered at pH 2.0 with 0.1 M sodium dihydrogen phosphate and pH 7.0 using disodium hydrogen phosphate. The results in Table VI show that at pH 2.0 neutral compounds were eluted with methylene chloride and basic compounds with methylamine in methanol, as expected. Recoveries averaged lower with the pH 2.0 phosphate buffer than when the sample was acidified with HCl. At pH 7.0 the weaker bases (aniline, quinoline, etc.) were not protonated, and therefore were eluted with the neutral group. The stronger bases (hexylamine, octylamine, etc.) remained protonated at this pH, and therefore were not eluted until the methylamine in methanol wash step. Thus an

Table VI. Recovery from 10 ml aqueous solution buffered at pH 2.0 and 7.0 with 0.1 M phosphate. All samples 5 ppm. Capacity of cation exchange resin is 0.89 meq/g.

Compound	CH_2Cl_2	$MeNH_2$ (pH=2)	CH_2Cl_2	$MeNH_2$ (pH=7)
pyridine	0	44	43	0
toluene	95	0	101	0
cyclohexyl amine	0	76	0	76
aniline	0	88	91	0
benzyl alcohol	93	0	98	0
benzonitrile	98	0	90	0
octyl alcohol	93	0	99	0
hexyl amine	0	91	0	90
octyl amine	0	91	0	90
p-ethyl phenol	92	0	91	0
o-hydroxy acetophenone	95	0	94	0
benzothiazole	94	0	100	0
quinoline	0	90	93	0
quinaldine	0	90	88	0
2,3-dimethyl quinoxaline	85	0	94	0
4-nitroacetophenone	75	0	86	0

additional group separation of aliphatic amines from aromatic amines and heterocyclic nitrogen compounds is possible.

This type of group separation within a group provides an additional mode of separation not previously demonstrated. It can be done by either of two methods, pH control in the loading step, or using a base of appropriate strength in the elution step.

Group Separation of Bases and Neutrals in Non-polar Solution

Volatile organic bases contained in a nonpolar solvent could be extracted as a group using this membrane. An example of this is shown in Figure 15 with a toluene solution containing trace amounts of piperidine, 3-picoline, and 2-ethyl pyridine. These early eluting bases cannot be separated from the large solvent peak by ordinary gas chromatographic methods. By adding sufficient HCl (10^{-4}M), the bases are protonated and thus retained by the sulfonic acid groups on the resin imbedded in the membrane. This acid concentration is an optimum and crucial to the success of the extraction. A higher concentration results in increased competition for the cation exchange sites, while a lower concentration appears to give incomplete protonation. Both of these scenarios lead to decreased extraction efficiency. After the toluene solution has passed through the membrane, the SPE column is blown dry. The bases are then eluted with

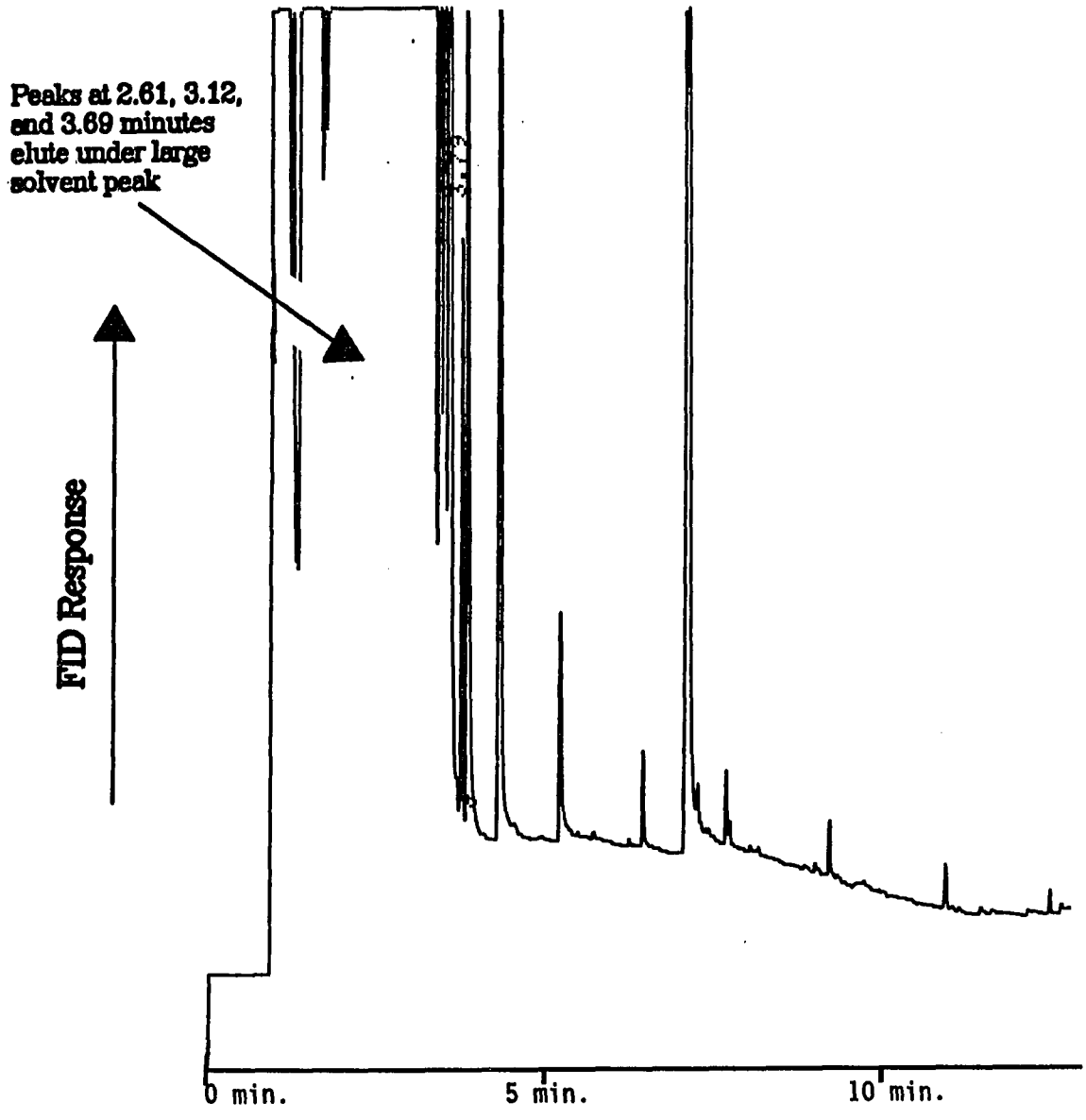


Figure 15. Gas Chromatogram of trace concentration (6 ppm) volatile bases in toluene (no extraction performed).

2M methyl amine in acetonitrile and determined by gas chromatography. An example of the resultant chromatogram is shown in Figure 16. The average recoveries of 4 trials is shown in Table VII.

This technique is also useful in extracting bases present at trace concentrations from complex non-aqueous mixtures . An example of this is shown in Figure 17. Five bases, benzyl amine, phenethyl amine, tributyl amine, quinoline, and quinaldine are present at 10 ppm in a toluene solution containing bromobenzene, 3-phenyl-propanol, octanol, decanol, dodecanol, and tetradecanol all at 1500 ppm. Direct analysis of the solution for the bases present by GC is impossible due to the large peaks present due to the various neutrals. But if the basic fraction is extracted by SPE, analysis by GC is possible. Two milliliters of this solution was acidified with HCl (0.0001M) and passed through the membrane at a flow rate of 1ml/min. The membrane was then allowed to blow dry with air pressure for a period of 1 minute. The bases were then eluted with 1 ml of 2M methyl amine in acetonitrile to which 0.1 ml of quinoxaline internal standard was added. Both the basic and neutral fractions were analyzed by GC. Excellent recoveries were obtained for the bases. The results are given in Tables VIII and IX.

Table VII. Percent Recovery of Basic compounds when extracted from toluene onto cation exchange membrane and eluted with methyl amine.

Basic compound	% Recovery
Piperidine	94.7
3-Picoline	100.2
2-Ethyl pyridine	96.5

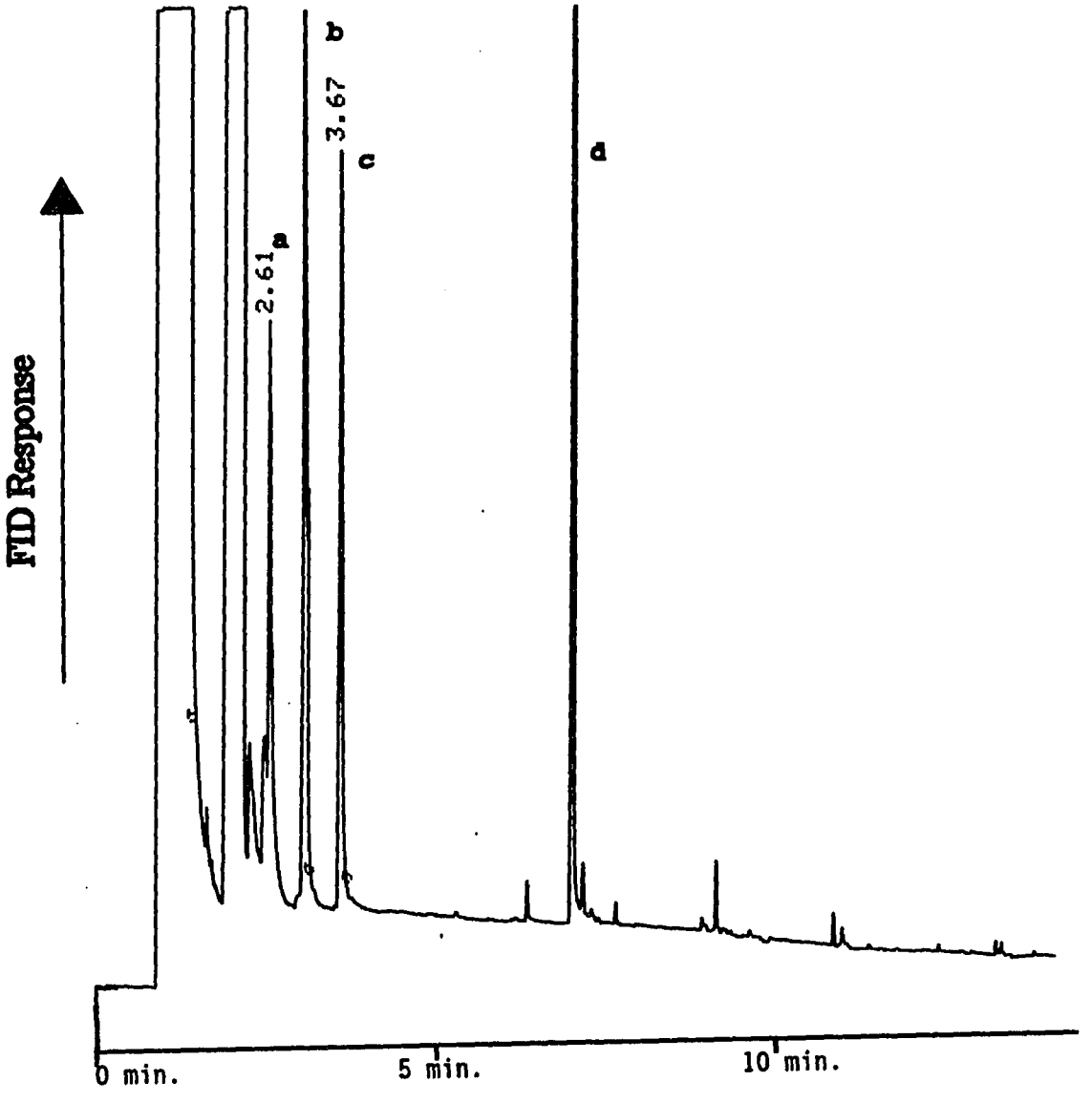


Figure 16. Gas chromatogram of early eluting bases after extraction from toluene. Bases are protonated by adding HCl to a mixture of 10^{-4}M, passed through the sulfonated membrane, blown dry, and eluted with 1 mL of 2M methyl amine in acetonirile. (a=piperidine, b=3-picoline, c=2-ethyl pyridine, d= quinoxaline (internal standard)).

Table VIII. Recoveries of neutral compounds from non-polar solution in base/neutral fractionation using sulfonated membrane.

neutral organic compounds	% recovery
octane	97
benzonitrile	104
nitrobenzene	98
isophorone	103
o-hydroxy propiophenone	97
butyl benzene	96
3-phenyl-1-propanol	100
phenetole	105

Table IX. Recoveries of bases extracted from non-polar solution with cation exchange membrane.

basic organic compounds	% recovery
benzyl amine	94
tributyl amine	95
phenethyl amine	99
quinoline	99
quinaldine	98
3-picoline	100
2-ethyl pyridine	102
isopropyl pyridine	95
N,N'-dimethyl pyridine	99

Examples of the chromatograms obtained are shown in Figures 17 and 18.

Ion Exchange Separation of Organic Bases in Non-aqueous Media

In an effort to better understand the forces present in ion exchange process in the uptake of organic bases by cation exchange resin, we studied the ion-exchange separation of organic bases in non-aqueous eluant. When an aqueous eluant is used in these processes, hydrophobic interactions play a significant role in the processes. We attempted to minimize the role of these hydrophobic effects by performing a chromatographic separation in a 100% alcohol eluant.

Very nice separations were achieved by this method. Figure 19 shows the separation of aniline, N'-methyl aniline, and N,N'-dimethyl aniline on just a 5 cm sulfonated column in a butanol/methanol eluant. By varying the chain length of the alcohol, we could control the relative polarity of the eluant. Table X shows the retention times of several bases in various alcohol mixtures. Looking at the smaller, more polar bases like pyridine and aniline, it is apparent from the retention times shown that in these eluants the separation is purely one of ion-exchange. The addition of a longer chain alcohol seems to play little role in the retention of the base when compared to the retention of these bases in methanol alone. Looking at larger bases, such as

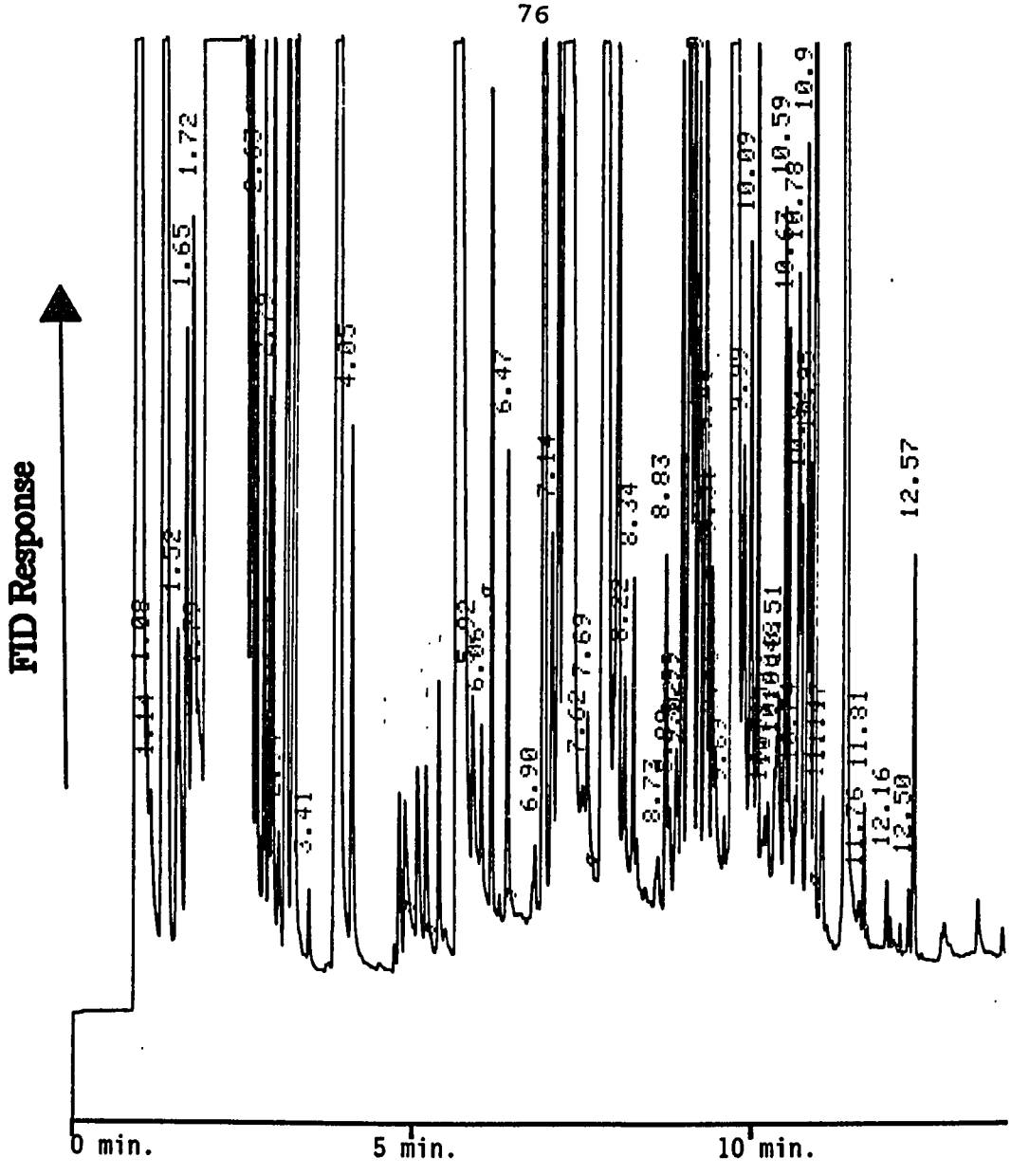


Figure 17. Mixture of bases and neutrals in toluene. Bromobenzene, 3-phenyl-propanol, 1-octanol, decanol, dodecanol, and tetradecanol present at ≈ 1500 ppm. Benzyl amine, phenethyl amine, tributyl amine, quinoline, and quinaldine present at 10 ppm.

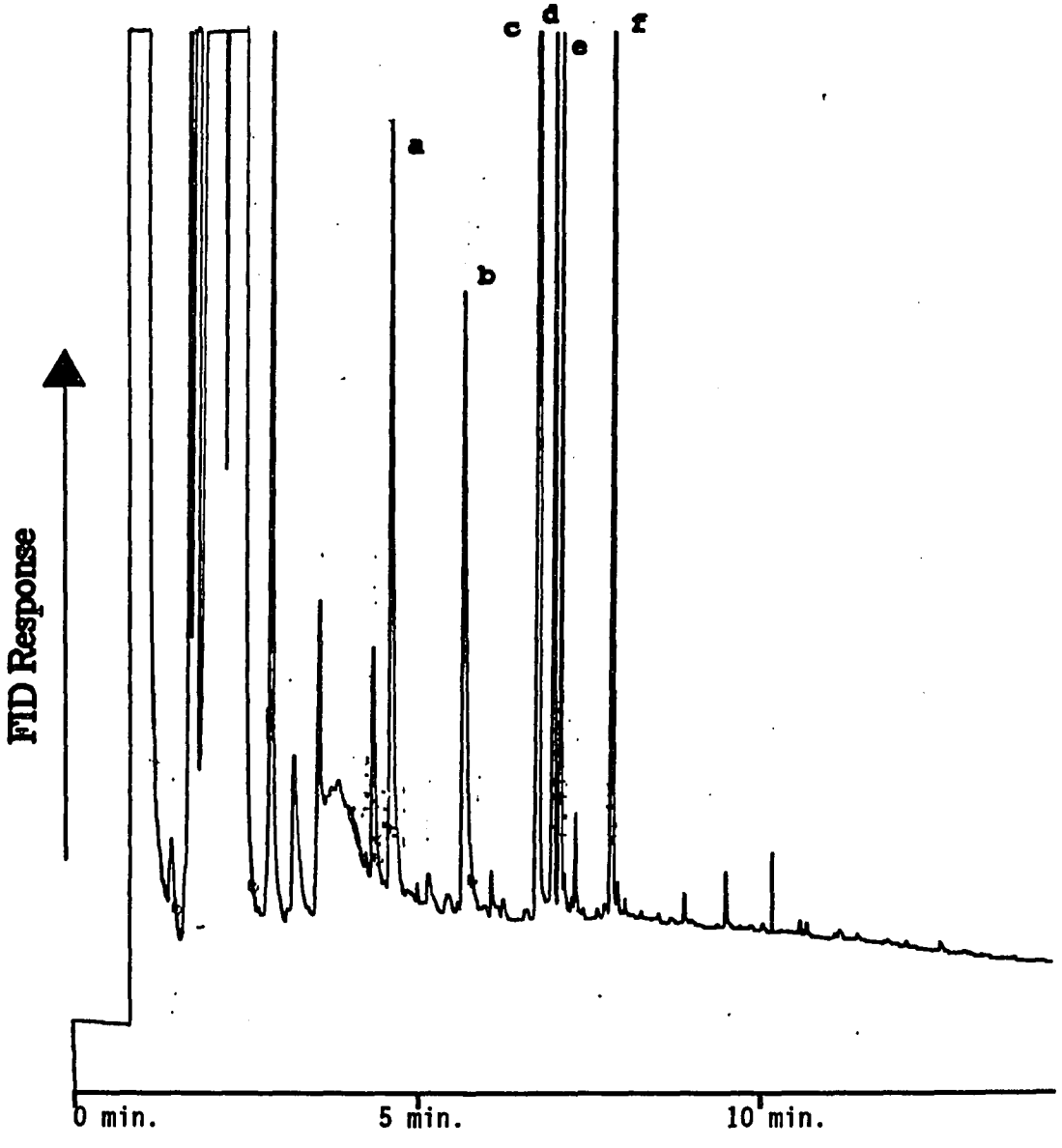


Figure 18. Gas chromatogram of bases extracted from 2 ml of base/neutral mixture in toluene (0.0001M HCl) and eluted with 1 ml of 2M methyl amine in acetonitrile. (a=benzyl amine, b=phenethyl amine, c=quinoxaline (int. Std.), d=tributyl amine, e=quinoline, f=quinaldine).

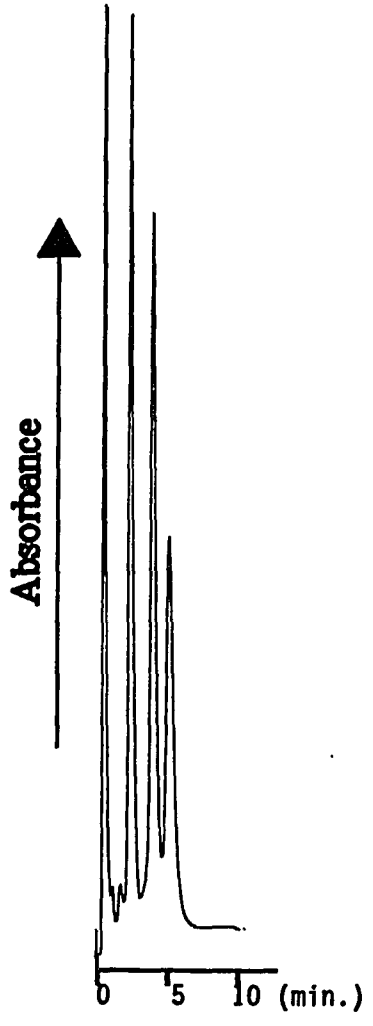


Figure 19. Chromatographic separation of aniline, N-methyl aniline, and N,N'-dimethyl aniline in 35/65% butanol/methanol eluant on 5 cm sulfonated column (0.15 meq/g). Flow rate = 1 ml/min.

Table X. Retention times of bases in non-aqueous solutions acidified with 0.02 M methanesulfonic acid.

organic base	(MeOH)	(65% MeOH/35% EtOH)	(65% MeOH/ 35% PropOH)	(65% MeOH /35% ButOH)
pyridine	3.76	3.87	3.82	3.78
quinoline	6.50	5.79	5.63	5.32
5,6-benzoquinoline	13.74	10.21	9.93	8.67
aniline	1.91	2.18	1.92	1.91
N-methyl aniline	3.12	3.94	3.55	3.50
N, N'-dimethyl aniline	6.12	5.55	4.71	4.65

N,N'-dimethyl aniline and 5,6-benzoquinoline, we see that the retention of these bases is reduced as the chain length of the alcohol increases. This indicates that hydrophobic interactions play a larger role in the separation of these bases. In fact, it most likely these interactions that control the actual separation of the bases. As these interactions are reduced, the retention times of similar bases like quinoline and 5,6-benzoquinoline become closer. This should allow for the optimization of these separations.

We also looked at the role of the acid in the separation. By varying the acid concentration of the eluent while keeping the composition of the eluent the same, we were able to plot log t_r, vs. log [acid]. In theory, the slope of this plot should be -1. Table XI shows that in most instances our results were very close to this value.

On-line Separation

In 1991 J. Sun and J. S. Fritz (38) demonstrated a method for the on-line extraction of basic compounds by placing a sulfonated polymeric resin in the injection port of a gas chromatograph. This sulfonated resin, placed in the injection liner and held in place with glass wool, extracted basic compounds from a sample solution while letting neutrals pass.

Table XI. Slopes of plots of log t_r' vs. log [acid]

organic base	slope (MeOH)	slope (65% MeOH/35% EtOH)	slope (65% MeOH/35% ButOH)
pyridine	-1.10	-1.00	-1.05
quinoline	-0.984	-0.988	-1.02
5,6-benzoquinoline	-1.00	-0.982	-1.06
aniline	-1.00	-0.982	-0.977
N-methyl aniline	-1.02	-1.02	-1.04
N, N'-dimethyl aniline	-1.02	-1.02	-1.08

The abstraction of basic compounds was essentially quantitative, but as yet no method has been developed for the elution of the adsorbed bases.

Simple modifications of this procedure allow for the group separation of bases and neutrals to be done on-line. A small 5 cm column was packed with sulfonated resin of a capacity of 0.15 meq/g. An ion exchange separation of organic bases can be performed on this column in a non-aqueous solution containing acid. In a non-aqueous solution, retention of the bases and neutrals by hydrophobic interaction can be minimized. In this case then, neutrals will be unretained and pass through the column in the void volume.

An example of this separation is shown with a mixture containing three neutrals and three bases in acetonitrile. This group separation is shown in Figure 20. A separation of these bases was performed in an eluent containing 65% methanol and 35% butanol acidified with 0.02M methanesulfonic acid. The mixture containing the neutrals and bases was injected onto the column. The first mL of eluant coming off the column was collected in a GC vial, an internal standard was added, and the eluant was then analyzed by a gas chromatograph.

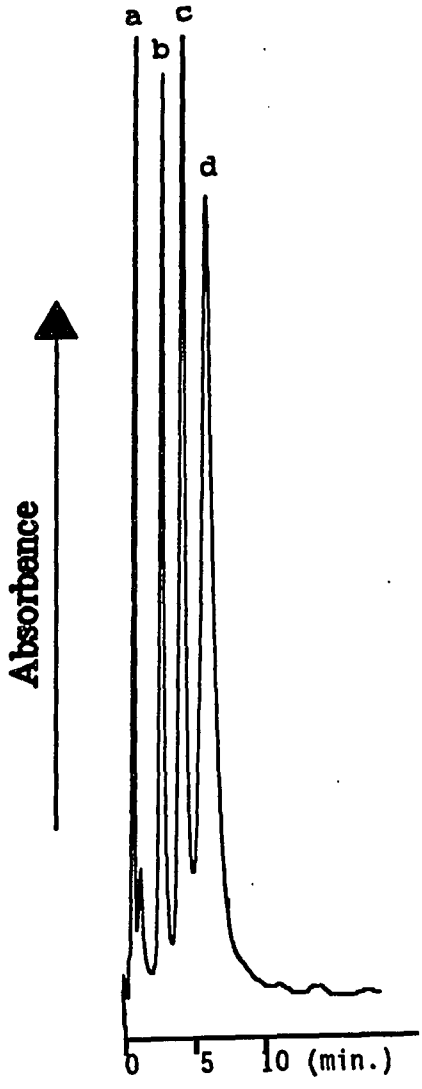


Figure 20. Chromatographic group separation of neutrals (a) and bases pyridine (b), quinoline (c), and benzoquinoline (d) in 35/65% butanol/methanol eluant on 5 cm sulfonated column (0.15 meq/g). Flow rate = 1 ml/min.

The gas chromatograph of these neutrals is shown in Figure 21. The recoveries of these neutrals were all very good, as shown in Table XII. This type of group separation could be easily automated and done with column switching.

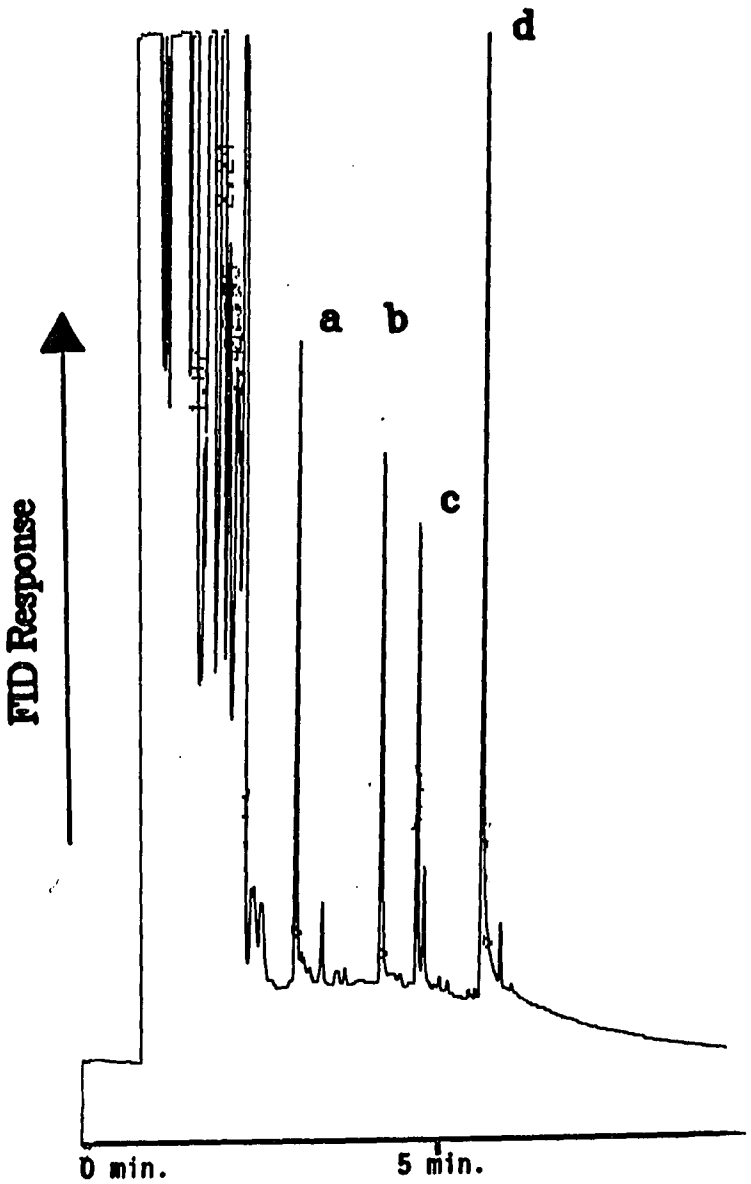


Figure 21. Gas chromatograph of neutral fraction collected in void volume in on-line separation. (a=benzonitrile, b=nitrobenzene, c=isophorone, d=quinoxaline (int. std.))

Table XII. Percentage Recoveries of neutral compounds in LC void volume.

Neutral Compound	% Recovery
Octane	98
Benzonitrile	100
Nitrobenzene	90
Isophorone	100

CONCLUSION

Fractionation of samples prior to chemical analysis is important for a number of reasons. It provides chemical information about analytes, simplifies complex samples, and provides an added separation mode to simplify analyses. In addition to a sample cleanup, it can allow for the identification and determination of trace components present in samples containing large amounts of other sample compounds.

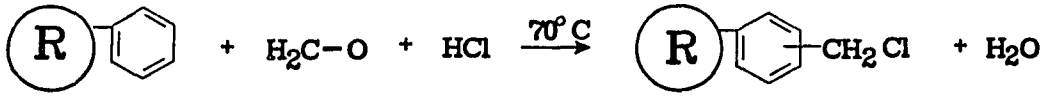
The fractionation schemes demonstrated here have proven to be effective with a variety of neutral compounds and both strong and weak bases. Fractionation is achieved for samples contained in polar and nonpolar matrices. It appears to be quite predictable, fast, and efficient. The recoveries of sample components are quantitative and reproducible.

PART III. ION EXCHANGE PRECONCENTRATION AND GROUP SEPARATION OF ACIDIC AND NEUTRAL COMPOUNDS

INTRODUCTION

As in Section II, we studied the use of partially derivatized ion exchange resins as a method of performing both preconcentration and group separation by SPE. Once again a medium capacity resin was used in a dual adsorption mode (see Figure 1). Neutral organic molecules in aqueous solution attach themselves hydrophobically at unreacted surface sites on the polymer, while anions will adhere electrostatically at the anion exchange sites.

The reaction used in the preparation of the resin was first described by Barron and Fritz in 1983 (41). The first step of this reaction is the chloromethylation step:



This is the step that is used to determine the capacity of the resin. This is done by controlling the reaction time at a constant temperature of 70°C. The second step, amination, is allowed to react to completion:

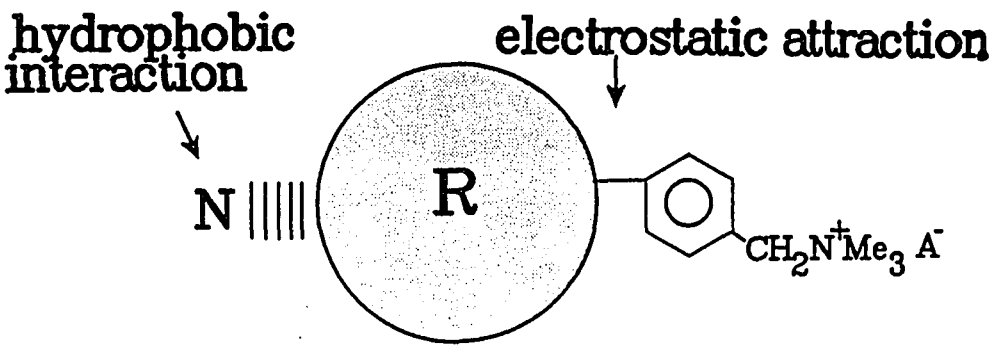


Figure 22. Diagram illustrating dual adsorption mode at single resin particle.

Once the sample analytes have been adsorbed onto the resin particle, a two-step elution process is again used to separately elute the neutral and acidic fractions. The neutrals are eluted with 1 ml of nonpolar solvent such as methylene chloride, and the acids are subsequently washed off the column with an acetonitrile or methanol solution containing an acid such as HCl or methanesulfonic acid. An outline for this scheme is shown in Figure 23.

This resin has also been used for the abstraction of organic acids from nonpolar matrices. This type of extraction is often vital, as the chromatographic methods of determination differ for volatile organics and carboxylic acids.

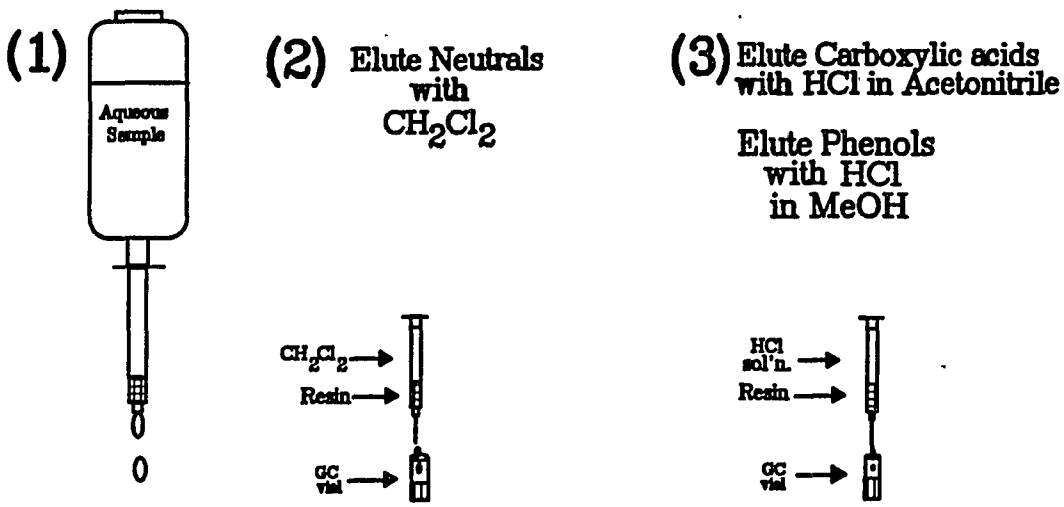


Figure 23. Concentration and two step elution scheme for group separation of neutral and acidic organic analytes.

EXPERIMENTAL

Reagents and Chemicals

The reagents used for the derivatisation of the ion-exchange resins were of analytical grade. Reagents used and analytes studied in SPE were >99% pure and used as obtained from Aldrich, Fisher, and Kodak. Laboratory distilled water was further purified using a Barnstead Nanopure II system (Sybron Barnstead, Boston, MA, USA).

The extractions were done using two different resins: a highly porous 40 μm cross-linked polystyrene resin, Amberchrome 161 (Supelco, Bellefonte, PA, USA), and an 8 μm PS/DVB resin, SRP 80 (Sarasep, Inc., Santa Clara, CA, USA).

The two step procedure for the preparation of the anion exchange resin is as follows. In an oil bath at 70°, 1 g of resin was wetted with 1 ml of glacial acetic acid. To this 25 ml of a 2.2 M solution of paraformaldehyde in concentrated hydrochloric acid was added. This chloromethylation step was allowed to proceed with slight stirring for 24 (for the Amberchrome resin) to 48 (for the SRP resin) hours. The resin was then filtered and rinsed with water and methanol. The resin was then aminated by adding 25 ml of 25% trimethylamine in ethanol and stirring at room temperaturee for 24 hours. The anion exchange product was then rinsed with methanol, water, and acetone.

The anion exchange capacity was determined by titration. A known mass of resin was put in its basic form by passing 5 ml of 1M NaOH over it. The resin was then rinsed with deionized water and tested with litmus paper to assure that excess NaOH was completely rinsed. Then, 5 ml of a standard HCl solution was passed through the resin. This was followed by another rinse with deionized water. The HCl and water rinse were collected and titrated with a standard NaOH solution. The capacity of the Amberchrome resin was determined to be approx. 0.9 meq/g. The capacity of the Sarasep resin was found to be 0.2 meq/g.

The apparatus for SPE consists of a 30 ml glass reservoir connected by a small adapter to the SPE column itself. The SPE columns were obtained from Varian (Harbor City, CA, USA). Approximately 100 mg of the resin was packed into the 55 by 6.5 mm I.D. columns. The Amberchrome resin was held in place above and below by 20 μm polyethylene frits. The SRP resin was held in place by cutting a circular disk out of a Rainin Nylon-66 filter of 0.45 μM pore size and sandwiching it between two of the polyethylene frits. The bed height of the amberchrome resin was about 15 mm, while the SRP was about 10 mm. The top of the reservoir was connected by a ground glass joint to an adjustable air

pressure source. The applied air pressure was used to control the flow rate of solutions through the SPE column. The columns were connected to the laboratory made reservoir by an adapter (Alltech Assoc., Deerfield, IL USA).

The concentrated phenol and neutral samples collected were analyzed using an HP 5880A gas chromatograph with a flame ionization detector, an HP 5880A Series level 4 integrator, and an HP 7637A automatic sample injector (Hewlett-Packard, Avondale, PA, USA). The capillary column used was a Supelco (Bellefonte, PA, USA) SPB-1.

The carboxylic acids were analyzed by HPLC using an LKB 2150 HPLC pump, an Applied Biosystem 783A UV absorbance detector, and a Shimadzu Chromatopac C-R3A integrator. The separation column was a 5 cm column packed with 5 μm sulfonated (0.6 meq/g) resin.

Procedure for SPE

Prior to initial use, the anion exchange columns were cleaned with acetonitrile and methanol. The columns were then treated with ca. 5 ml of NaOH solution to ensure the resin was in the basic form.

The sample solutions were prepared by spiking a dilute methanol solution of several neutral and acidic compounds to the deionized water such that a final concentration of

ca. 0.5 to 5 ppm is attained. The pH of this solution was then raised to ca. 11 with NaOH. The sample was then put in the reservoir and passed through the resin at a flow rate of approximately 1 ml/min. The column and reservoir were then rinsed with about 5 ml of water.

The column was rinsed with 1 ml of methylene chloride to elute the neutral fraction. This fraction was collected in a GC vial and spiked with 0.1 ml of an quinoxaline or toluene internal standard solution. The vial was capped, mixed with an orbital stirrer, and analyzed by GC. A 1 μl sample was injected with a split ratio of 1:40. Helium carrier gas was used at a flow rate of 1 ml/min. The oven temperature was held initially at 50°C for two minutes, then ramped at 15°C/min to a final temperature of 225°C. A flame ionization detector was used. The acid fraction was then eluted with 1 ml of 0.1M HCl in methanol. When the acid fraction contained phenols, the analysis was performed by GC as described for the neutral fraction. Recoveries were calculated by comparison of relative peak area to that of samples prepared and not subjected to SPE.

Samples containing carboxylic acids were eluted with 1 mL of 2M HCl in methanol and analyzed by HPLC. A 5 μl aliquot of the sample was injected onto a 5 cm laboratory

prepared column of 1 meq/g sulfonated 5 μm PS/DVB resin. Separations were performed using an aqueous eluant of 10% acetonitrile, 1 to 2% butanol, and 1 mM H_2SO_4. Quantitation was achieved by comparison of peak areas to known standards.

RESULTS AND DISCUSSION

Group Separation of Acids and Neutrals

In the previous section it was illustrated that partially sulfonated resins still have the ability to retain neutral molecules by hydrophobic interactions at unreacted sites on the resin particle. This ability can lead to adsorption in a dual mode, hydrophobic and electrostatic attractions thus allowing for group separations. In this section the same principles outlined in the base/neutral separations are used with anion exchange resins to perform acid/neutral separations. The pH of the sample solution is raised to a basic pH with NaOH to ensure that the acids are present as anions and thus retained at the ion exchange sites.

Figure 24 shows a gas chromatogram of the neutral fraction obtained when concentrated and eluted with HCl in methanol. The average recoveries (n = 5) of a large number of neutrals are shown in Table XIII. In most instances, the recoveries of the neutrals are good (> 90 %).

Phenols are eluted with 1 ml 0.1 M HCl in methanol. It has been shown (Chen and Fritz, unpublished results) that when relatively small acid concentrations are injected onto a GC column in small volumes (≈ 1 μl), the column shows no

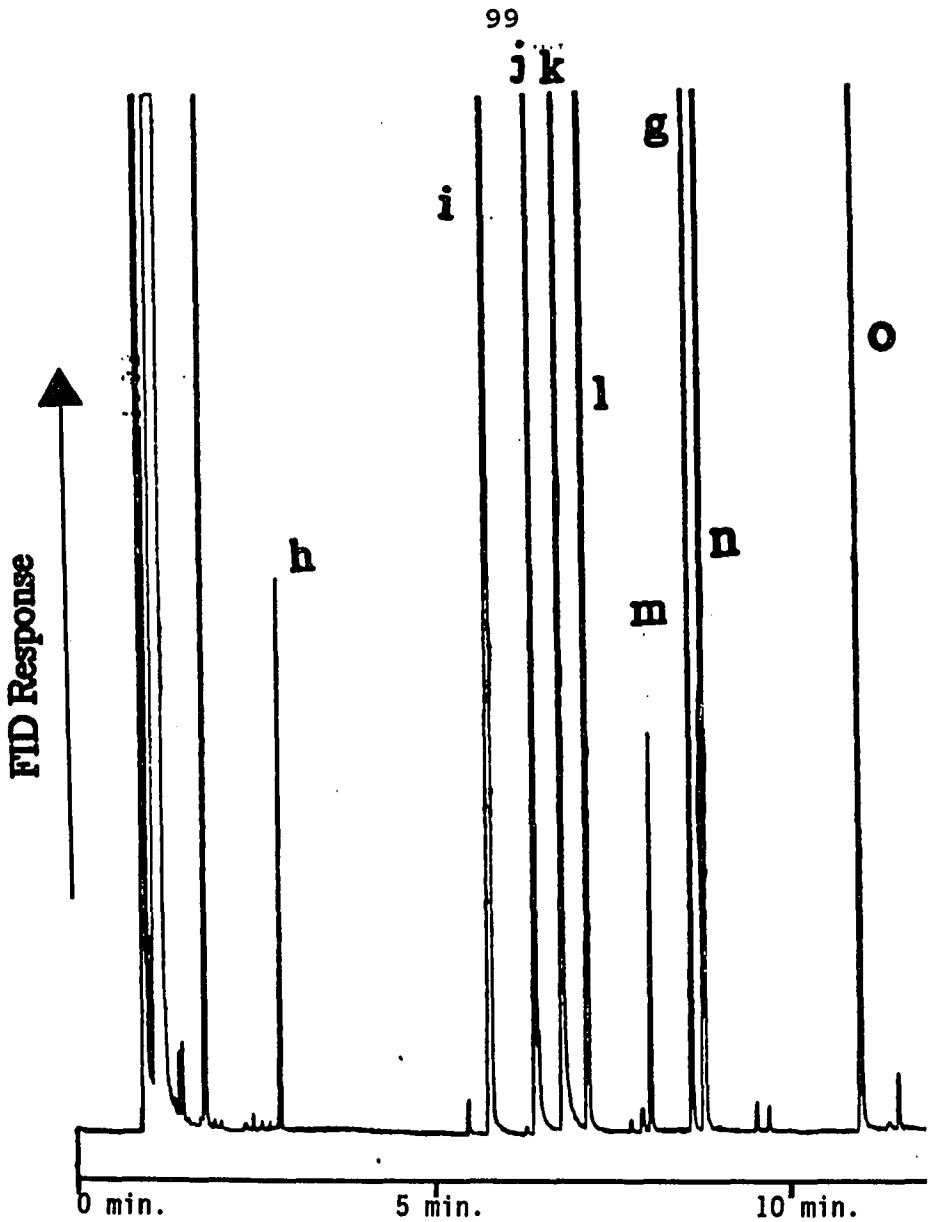


Figure 24. Gas chromatogram of neutral fraction concentrated on anion exchange resin and eluted with methylene chloride. (h=toluene, i=benzyl alcohol, j=benzonitrile, k=octyl aldehyde, l=nitrobenzene, m=o-hydroxy acetophenone, n=benzothiazole, o=p-nitroacetophenone, g=quinoxaline (int. std.))

Table XIII. Recoveries of neutrals (0.5-5 ppm) from 20 ml aqueous solutions using anion exchange resins.

Compound	% Recovery
Ethyl butyrate	101
Chlorobenzene	87
Cyclohexanol	100
Bromohexane	90
Salicyl aldehyde	99
Octanol	100
Nonyl aldehyde	100
Triethyl orthopropionate	84
Decanol	96
Benzyl alcohol	93
Octyl aldehyde	92
Nitrobenzene	96
Benzothiazole	103
Toluene	93
Anisole	96

significant degradation over time. The small amount of acid present in these small injection volumes seems to be adsorbed onto the glass wool and silica contained in the glass liner of the injection port. This allows for the direct injection of the eluted acid fraction onto the GC column. Figure 25 shows the chromatogram obtained when the phenolic acid fraction is analyzed by GC. The resulting recoveries are shown in Table XIV.

When carboxylic acids were studied, analysis could not be performed by GC, due to the limited volatility of the underivatized sample. In this case the retained acids were quantified by ion exclusion chromatography on a laboratory prepared 5 cm sulfonated PS/DVB column. The carboxylic acids were eluted from the SPE column with 1 ml of 1M HCl in acetonitrile. This eluate was then injected directly onto the ion exclusion column for analysis. An example of the separation performed is shown in Figure 26 and recoveries given in Table XV.

Group Separation of Acids and Neutrals in Non-polar Media

Carboxylic acids can also be extracted from nonpolar solutions in a fashion similar to that illustrated with bases in Section II. Carboxylic acids, being relatively involatile, cannot be injected onto a GC column without significant effects on the separation. This makes a method

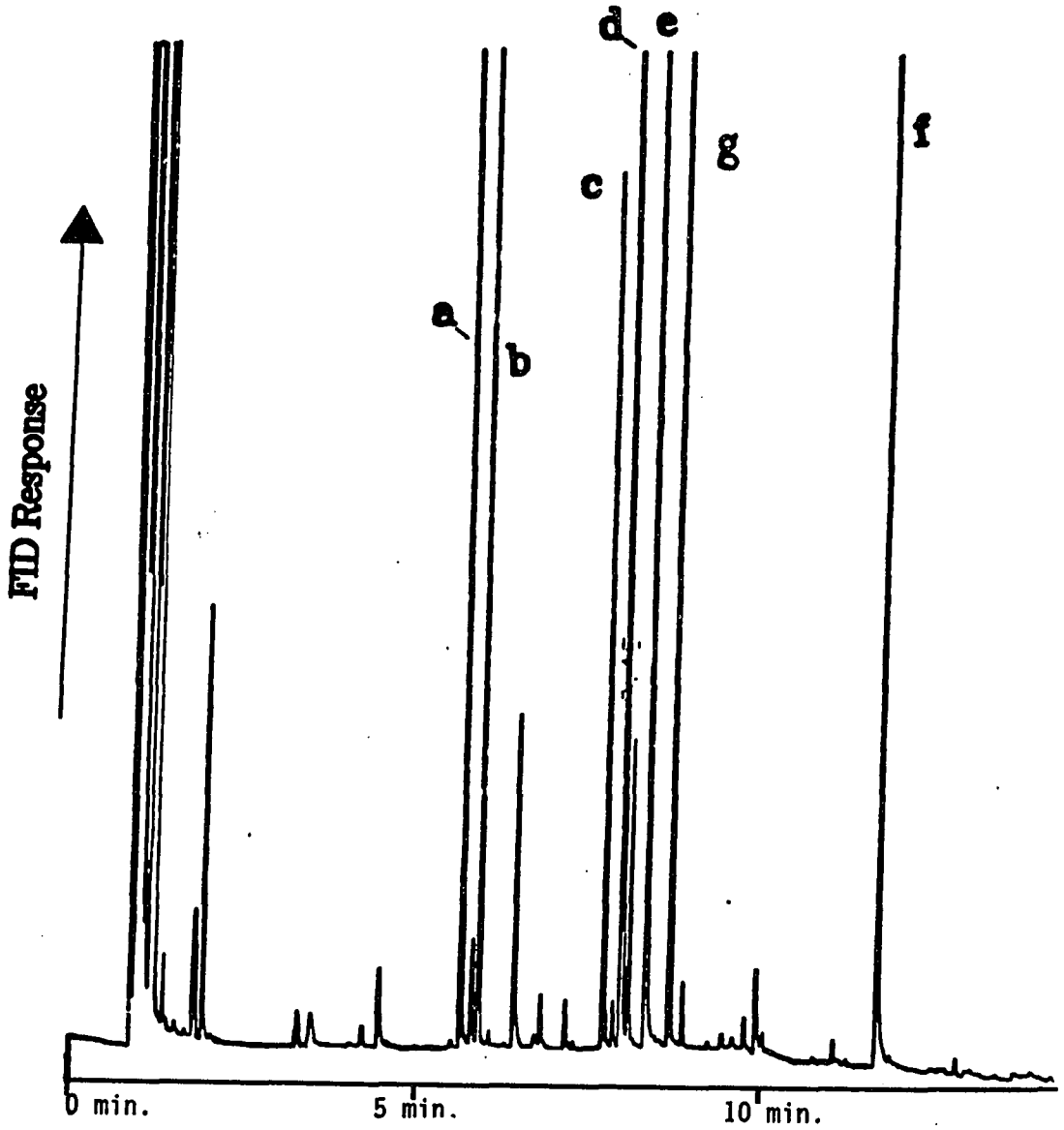


Figure 25. Gas chromatogram of acid (phenolic) fraction concentrated on anion exchange resin and eluted with 0.1M HCl in methanol. (a=phenol, b=2-chlorophenol, c=4-chlorophenol, d=p-ethylphenol, e=o-nitrophenol, f=p-nitrophenol, g=quinoxaline (int. std.))

Table XIV. Percent recoveries of phenols (5 ppm) from 20 ml aqueous solution using anion exchange resin.

Compound	% Recovery
Phenol	87
2-Chlorophenol	95
4-Chlorophenol	99
o-Nitrophenol	99
m-Nitrophenol	100
p-Nitrophenol	94
p-Cresol	98
2,5-Dimethyl phenol	94
p-Isopropyl phenol	100
p-sec-Butyl phenol	100
p-Ethyl phenol	100

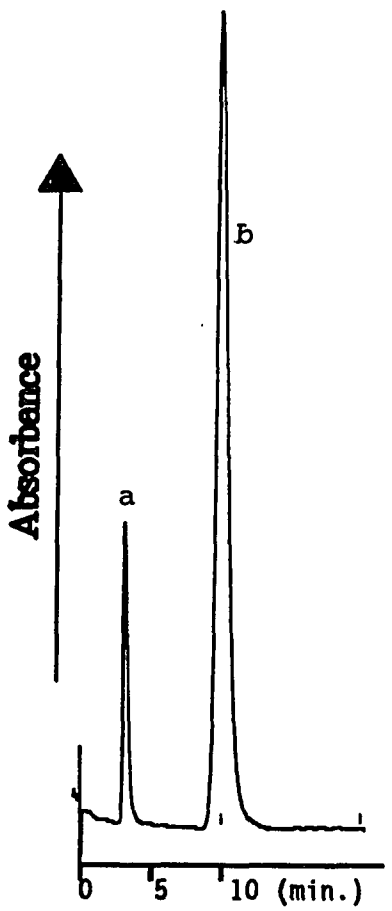


Figure 26. Liquid chromatogram of acidic (carboxylic acid) fraction concentrated on anion exchange resin and eluted with 0.1M HCl in methanol. Acids shown are (a) hydroxy benzoic acid and (b) benzoic acid.

Table XV. Percent recoveries of carboxylic acids (5-10ppm) phenols from aqueous solution using anion exchange resin.

Carboxylic acid	% Recovery
Hydroxy benzoic acid	99
Benzoic acid	96
p-Nitrobenzoic acid	99
1,2,4-Benzene tricarboxylic acid	105
Salicyl hydroxamic acid	102
Isonicotinic acid	95
2,4-Dihydroxy benzoic acid	99
Isophthalic acid	100
Phthalic acid	100

for their removal from solution to be analyzed by GC important. To 2 ml of toluenesolution containing a mix of neutrals and carboxylic acids

0.1 ml of 1M methyl amine was added in order to convert the acids to anions. This solution was then passed through an SPE column containing ≈ 10 mg of the 5 μm anion exchange resin to remove the acids. The neutral solution was then collected and analyzed by GC (Figure 27). The abstracted acids were then eluted from the column with 1 M HCl in methanol and analyzed by Ion Exclusion Chromatography as before. The recoveries of the neutrals and acids studied are shown in Tables XVI and XVII.

Though this type of separation may seem trivial, the presence of non-volatile carboxylic acids in samples to be analyzed by GC can be quite disastrous. Being semi-volatile, these acids become trapped at the head of a column, effectively ruining the column by causing problems with future separations, necessitating the removal of that portion of the column.

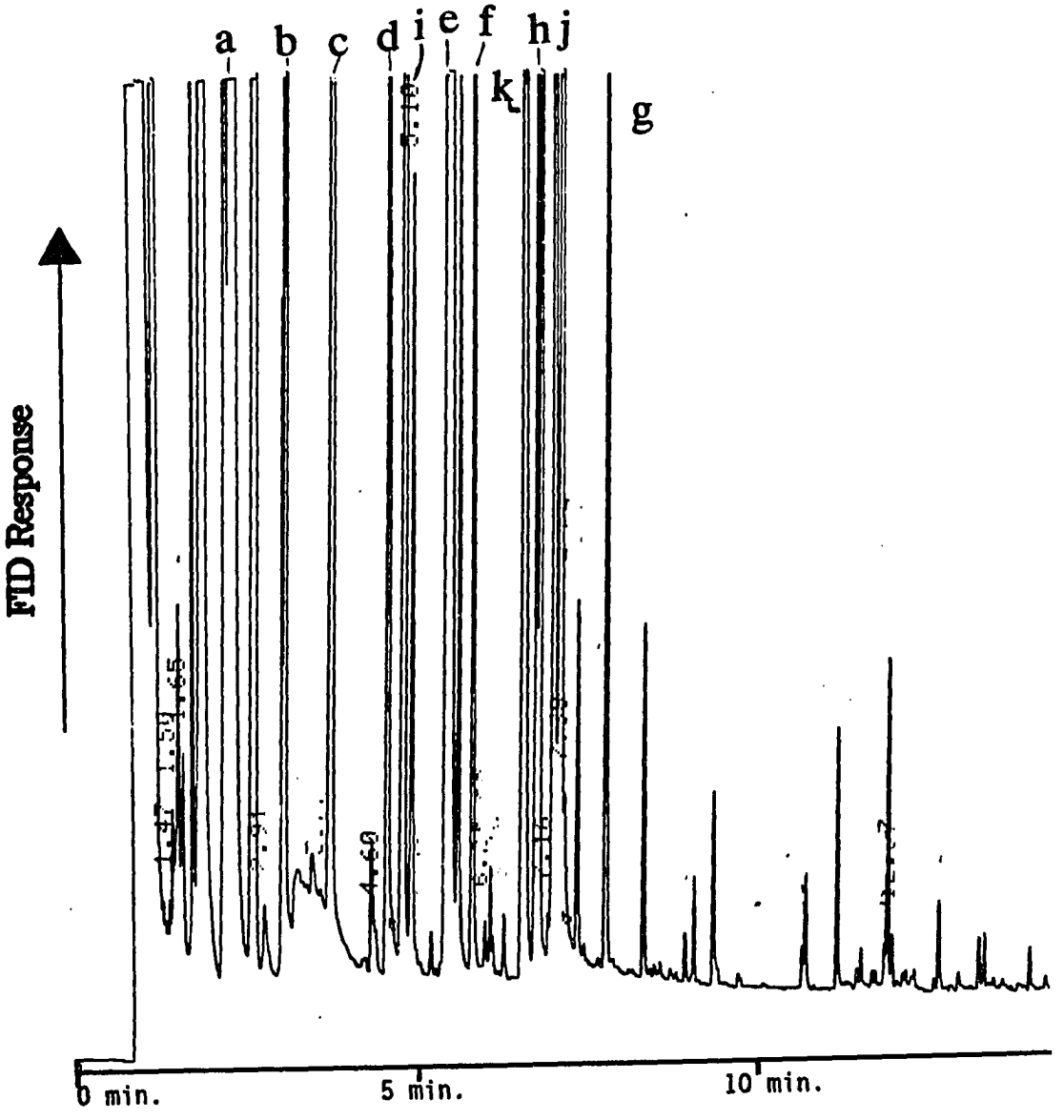


Figure 27. Gas chromatogram of neutral fraction after abstraction of carboxylic acids with anion exchange resin. (a=ethyl butyrate, b= chlorobenzene, c=cyclohexanol, d=bromohexane, e=salicyl aldehyde, f=octanol, g=nonyl aldehyde, h=quinoxaline (int. std.), i= triethyl orthopropionate, j=decanol, and octyl aldehyde).

Table XVI. Recoveries of neutrals (100 ppm) in 2 ml acid/neutral group separation from toluene using anion exchange resin. Analysis by GC. (n = 5)

Neutral Compound	% Recovery	Std. Deviation
Ethyl butyrate	103	6
Chlorobenzene	97	5
Cyclohexanol	99	4
Bromohexane	98	3
Triethyl orthopropionate	101	2
Salicyl aldehyde	94	2
Octyl aldehyde	107	10
Decanol	99	5
Nonyl aldehyde	98	3
Octanol	99	3

Table XVII. Recoveries of carboxylic acids (50 ppm) from 2 ml acid/neutral mixture in toluene using anion exchange resin. Acids eluted with HCl in acetonitrile and analyzed by ion exclusion chromatography. (n=5)

Carboxylic acid	% Recovery	Std. Deviation
Benzoic acid	97	5
2-Hydroxy benzoic acid	98	1
1,2,4-Benzene tricarboxylic acid	99	1
1,2,4,5-Tetracarboxylic acid	99	6
2,4-Dihydroxy benzoic acid	101	2
Phthalic acid	98	5
Isonicotinic acid	98	1

CONCLUSION

This type of concentration/group separation has many possible applications with both environmental and biological samples. Examples might include the cleanup and isolation of acidic drugs, phenolic pollutants, and humic acids. The group separation gives additional chemical information and provides another mode of separational ability. It allows complex samples containing compounds that must be analyzed by different methods to be separated prior to analysis.

The methods demonstrated were quick and precise, and the possibility of automation give them a bright future. Results were good for most compounds and reproducible. On-line separations, demonstrated in the last section for the separation of bases and neutrals, should be applicable in the separation of neutrals and acids as well.

GENERAL CONCLUSION

Solid Phase Extraction (SPE) is fast becoming the preferred method for cleanup and concentration of liquid samples prior to chemical analysis. An increasing number of papers are published using these techniques for specific problems, and a large number of vendors are marketing disposable prepacked SPE tubes. Companies such as Bio-Rad (Richmond, CA), J. T. Baker (Phillipsburg, N.J.), and Supelco, Inc. (Bellefonte, PA) have all published guides to applications of their SPE apparatus. As advances are made in the polymeric particles used, SPE may replace liquid/liquid extraction for routine analysis. The benefits of SPE by way of decreased solvent use, less labor involved, and higher extraction efficiencies give this technique promise.

One of the major concerns for the analytical chemist in today's world is the environment. As the list of chemical entities to be monitored increases, sample preparation techniques specific to certain classes of compounds become more important. The ability to selectively abstract and fractionate a class of pollutants can ease the demands on the analytical instrument. As the levels of these pollutants are often quite small with regards to other entities in the matrix, the ability to abstract and concentrate begins doubly important. Often times the ill effects of pollutants are

felt even at sub ppb levels. The old adage prevalent in industry with regard to the discharge of wastes, "dilution is the solution to pollution", may not always be true.

SPE is also finding uses in clinical laboratories. An increased awareness of the problems brought on by drugs in the workplace has created a need for drug testing in industry, and techniques are needed to detect low levels of these drugs in biological fluids. A sample prep technique which can extract, concentrate, and fractionate drugs in urine or blood samples is needed. SPE is starting to fill this void.

The acetyl membrane demonstrated in this work shows promise in the environmental analysis of polar organics in aqueous solutions. Phenolic wastes have often been a thorn in the side of SPE. Being relatively hydrophilic, they give low recoveries when extracted with Si-C_{18} or underivatized polymeric resins. By chemically modifying the surface of the polymeric resin with a polar acetyl group, the resin was made more amenable to phenols present in water. This is supported by the fact that the acetyl resin showed good efficiency in the abstraction of phenols from deionized, tap, and river water samples.

By sulfonating a polymeric resin to produce a cation exchange resin, we were able to accomplish two things. The

addition of the sulfonic acid group allows for the uptake of cations electrostatically, while allowing for the adsorption of neutrals at underivatized sites on the resin. The sulfonic acid groups on the resin increase the hydrophilicity of the resin, creating a more efficient interaction between solution and surface. The recoveries of both neutrals and protonated bases were quite good, and a complete group separation was performed. This type of separation is important in complicated sample mixtures and in the separation of classes of drugs. The ability to selectively abstract a species according to the pK_b of the analyte can be a useful tool in the analysis of trace components in complex mixtures. The separation has been developed for both polar and apolar solutions, extending its application to all liquid samples. An on-line group separation has also been demonstrated. This type of work has a future in process analytical chemistry and the analytical laboratory. Any process that reduces the manual labor not only increases laboratory efficiency, but also cuts out analysis steps which may introduce errors in the final analysis.

The introduction of a quaternary ammonium group to the polymeric resin allows for the group separation of acid and neutrals. This type of separation is done in both clinical (acidic and neutral drugs) and environmental (phenols and

humic acids) analysis. The extraction of acids is especially important prior to analysis by gas chromatography, as the majority of acids are involatile and must be analyzed by alterior methods. Additionally, exposure to acids decreases the life of a GC column significantly. In the future, this type of abstraction could be done on-line to prevent the fouling of columns by tainted or acidic samples.

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Chapter 12

Principles and practice of solid-phase extraction

Colin F. Poole

12.1 INTRODUCTION

Solid-phase extraction (SPE) is a method used for the isolation and concentration of selected analytes from a gas, fluid or liquid (usually) flowing sample stream by their transfer to and retention (sorption) on a solid phase. The solid phase is then isolated from the sample and the analytes recovered by elution using a liquid or fluid, or by thermal desorption into the gas phase. Occasionally the sampling step is performed by suspension of a suitable SPE device in a liquid, fluid or gas phase long enough to reach equilibrium with the sample, or some other reproducible end-point, followed by mechanical separation of the solid phase from the sample. The analytes are then recovered by elution or thermal desorption, as before. The principal goals of SPE are trace enrichment (concentration), matrix simplification (sample clean-up) and medium exchange (transfer from the sample matrix to a different solvent or to the gas phase). SPE was initially developed as a complement or replacement for liquid–liquid extraction (LLE). It is now the most common sampling technique in many areas of chemistry, including environmental, pharmaceutical, clinical, food and industrial chemistry. Over time various sampling formats and sorbents have been developed to facilitate the convenient processing of different sample types and to extend the scope of the method. A high level of automation is also possible using robotics or on-line interfaces to separation and spectroscopic instruments. Full system integration for unattended extraction, separation and detection is possible although flexible, inexpensive manual sample processing remains the more common practice in most laboratories.

In this chapter I will describe the principles and practice of SPE for the analysis of liquid samples using cartridge and disc devices. SPE using cartridges for atmospheric sampling and solid-phase microextraction, in-tube solid-phase microextraction and stir bar extraction are discussed elsewhere in this book, while specific applications of SPE can be found in the applications section and in Refs. [1–6].

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12.2 EVOLUTION OF SOLID-PHASE EXTRACTION

12.2.1 Historical development

Fortuitous application of the SPE principle predates written history, but its general use in laboratory and field sciences is more recent. Inorganic oxide columns were used from the 1930s for the clean-up of samples in organic solvents, but this was rarely considered an extraction process. Probably the first significant analytical application of SPE was the use of metal pipes filled with activated carbon in the early 1950s for the isolation of organic contaminants from various water sources [7]. These studies were performed to isolate sufficient material for structural identification with the relatively insensitive instruments available at

TABLE 12.1

Milestones in the development of solid-phase extraction

Early 1950s	Pipes filled with activated charcoal used to isolate microcontaminants from surface waters.
Late 1960s	Macroreticular porous polymers introduced as a replacement for active charcoal. Standardized protocols developed using small columns. Applications to biological fluids and air sampling developed.
Mid 1970s	Silica-based chemically bonded phases used in solid-phase extraction and eventually dominate the practice of solid-phase extraction. Disposable cartridges introduced in 1978 and the open syringe barrel format in 1979.
Early 1980s	Short precolumns introduced for automated solid-phase extraction and liquid chromatography. This did not become a routine technique until the early 1990s.
Mid 1980s	Start of the development of sorbents with improved molecular recognition properties. Mixed mode (reversed phase/ion exchange) sorbents introduced for improved isolation of drugs from biological fluids (1985). Restricted access materials introduced for the selective isolation of drugs in biological fluids without prior removal of proteins (1985). Immunosorbents developed to take advantage of the specificity of antibodies for the isolation of target analytes from complex mixtures (Biomedical applications developed in the early 1980s followed by specific procedures for food contaminants and then environmental applications by the mid-1990s). Silica or other supports with tethered mixed oxygen-nitrogen donor cryptands introduced for the selective isolation of metals by a molecular recognition mechanism (1988).
1989	Matrix dispersion introduced as a sample processing technique.
1989	Particle-loaded membranes for disc extraction introduced. Soon followed by particle-embedded glass fiber discs and laminar discs. Starts the development of disc technology as a complement to cartridge sampling devices.
1992	Solid-phase microextraction is brought to commercial fruition.
1994	Molecularly imprinted polymers introduced as synthetic analogs of immunosorbants.
Late 1990s	Multiwell plates for high throughput sample processing introduced. Stir bar extraction (1999) and in-tube solid-phase microextraction (early work in the 1980s but no general acceptance until late 1990s as an interface for microcolumn liquid and gas chromatography) continue the development of new formats for convenient sampling using a solid-phase sorption mechanism.

that time and for toxicity assessment. The large volume of water generally sampled (>1000 liters over several days) meant that LLE procedures were inappropriate or difficult to implement. Since then a number of different sorbents and formats for SPE have been introduced, as summarized in Table 12.1.

The heterogeneous nature of activated carbons available at that time hampered any extensive development of SPE. These materials had poorly defined properties characterized by variable and weak adsorption of some analytes, irreversible adsorption and low recovery of others, and occasional chemical modification of some analytes [8]. The introduction of poly(styrene-divinylbenzene) and poly(methacrylate) macroreticular porous polymer beads, prepared by suspension polymerization, in the late 1960s was responsible for rekindling interest in the SPE of organic contaminants from water and for extending the scope of SPE to air sampling and the analysis of biological fluids [6,7,9–13]. Polymer sorbents played a key role in the development of purge-and-trap techniques for the routine determination of volatile organic compounds in water [14,15]. These chemically inert sorbents had reasonable mechanical strength (at least compared to gels), with a large surface area, high sample capacity, low water retention and provided high sample recoveries by either solvent elution or thermal desorption. Compared with carbon analyte recovery was generally better and irreversible adsorption and catalytic activity greatly diminished. Standardized sampling procedures using small columns, early versions of modern cartridge devices, were introduced but the problem of extract contamination by sorbent impurities remained a persistent problem.

SPE for liquid samples became a widely used laboratory technique in the early 1980s with the introduction of disposable cartridges containing silica-based chemically bonded sorbents of a suitable size for sample processing by gentle suction. Typical cartridge devices consist of short columns (generally an open syringe barrel) containing a sorbent with a nominal particle size of 50–60 μm, packed between porous plastic or metal frits (Fig. 12.1) [1–4,6,16–19]. A large number of non-selective and selective sorbents (including restricted access media, mixed-mode sorbents, immunosorbents, molecularly imprinted polymers, chemically bonded cryptands, etc.) are available today providing for the diverse application base of modern SPE. Low volume cartridge or precolumn devices are the basis of on-line hyphenated systems (SPE-LC, SPE-GC) for

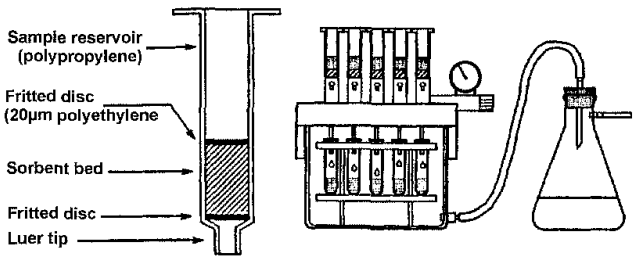


Fig. 12.1. Schematic diagram showing the typical construction of a solid-phase extraction cartridge and a vacuum manifold for parallel sample processing.

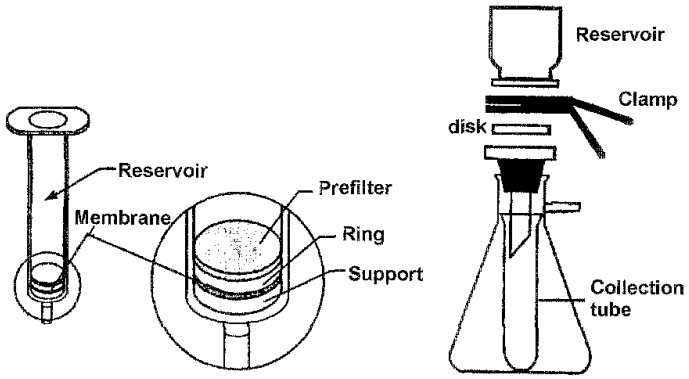


Fig. 12.2. Typical cartridge and vacuum filtration formats for solid-phase extraction using discs.

integrated sample processing and separation that matured into robust practical systems in the mid-1990s [6,19–23].

SPE discs were developed initially to provide higher sample processing rates for large sample volumes and to minimize plugging by suspended particles and matrix components [24–29]. Since small-diameter discs are easily prepared, discs are also favored for handling small-volume samples as well. At least three different disc formats are offered today. Particle-loaded membranes contain sorbent particles of 8–12-μm diameter immobilized in a web of short poly(tetrafluoroethylene) (PTFE) fibrils [24–27]. These are formed into 0.5-mm thick discs of various sizes containing about 90% by weight of sorbent. The discs are flexible and superficially resemble filter paper discs. They are used with some supporting structure such as a porous glass or plastic support (Fig. 12.2). Particle-loaded membranes are also available in a conventional cartridge format. In this case, the sorbent bed contains particles of a larger size, about 50 μm, in a thicker disc of about 1.0 mm, sealed into the base of an open syringe barrel. These discs have an integral poly(propylene) prefilter attached to their top surface. Particle-embedded glass fiber discs contain particles of about 10–30 μm in diameter woven into a glass fiber-supporting matrix [28,29]. Small diameter discs are self-supporting but larger discs require a supporting structure similar to the particle-loaded membranes. Particle-embedded glass fiber discs are also available with an integral prefilter attached to their top surface. Laminar discs contain 10-μm sorbent particles in a consolidated 0.5- or 1.0-mm thick bed (usually) retained by two glass fiber filters held in place by screens and a retaining ring in a preassembled cartridge [30]. Disc technology has contributed directly to the automation of SPE through development of the multiwell extraction plate (Fig. 12.3), which is used for isolation and sample clean-up in high-throughput screening techniques, such as combinatorial chemistry and drug development [31–36].

12.2.2 As a replacement for liquid–liquid extraction

SPE was initially considered as a replacement for liquid–liquid extraction (LLE). Conventional LLE is labor intensive, difficult to automate, and is frequently

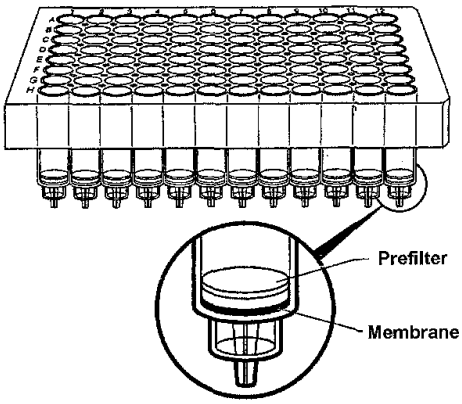


Fig. 12.3. Multiwell plate for automated SPE. (From Ref. [31]; copyright Elsevier).

plagued by practical problems, such as emulsion formation. In addition, it consumes relatively large volumes of high purity solvents with expensive disposal requirements. In contrast SPE benefits from low intrinsic costs, shorter processing times, low solvent consumption and simpler processing procedures. SPE methods are easier to automate using robotics, centrifugal or special purpose sequential or parallel flow processing units that simultaneously extract and prepare samples for separation. In addition, SPE has favorable properties for field sampling, eliminating the need to transport and store bulk samples for processing by the receiving laboratory.

SPE techniques have their own, although different, problems to those of LLE. The surface chemistry, and therefore sorption properties, of solid phases are not as reproducible as solvent properties. Silica-based, siloxane-bonded sorbents retain basic compounds by a mixed retention mechanism involving low-selectivity sorption by the bonded phase and ion-exchange interactions with accessible dissociated silanol groups of the silica substrate. The mixed retention mechanism can interfere in analyte recovery, since elution with neutral solvents is ineffective for displacing ionically bound analytes, and the contribution of ionic binding to retention can vary for different sorbents. Solid phases tend to have a higher level of contamination by manufacturing and packaging materials than is the case for solvents. The chemical background from impurities may interfere in the subsequent determination of the analytes. Sample processing problems in SPE related to the limited sorption capacity of sorbents and analyte displacement or plugging of sorbent pores by matrix components, easily pass unnoticed, resulting in changes in analyte recovery. Sample overload and displacement are more important for SPE than LLE because of the limited sorption capacity of sorbents compared with the solvation properties of liquids.

A large number of factors have to be considered when selecting SPE or LLE for a particular problem. These include economic as well as technical features, whether manual or automated operation is to be pursued and the matrix chemistry and quantity. It is quite likely that a LLE or SPE method can be developed for the same problem and a final selection is based on a feasibility

study where one technique emerges as a preferred choice from consideration of the positive and negative features of each method. Increasingly as external factors dictate a reduction in the use of popular solvents this has tended to favor SPE. Also, as laboratories look at approaches to increase sample throughput and reduce labor costs, this has also tended to favor SPE methods. For field sampling SPE provides a simpler approach than solvent extraction methods.

12.2.3 Choice of cartridge or disc formats

Cartridge and disc devices use the same sorbent technology and are distinguished only by differences in format. Cartridges are easily prepared in the laboratory while discs, so far, have only been produced in a manufacturing setting. Discs, therefore, are only available in a limited range of sorbent chemistries, largely dictated by market forces. Also, it is easier to scale-up cartridge devices to handle large sample loads and to perform sample clean-up than it is for discs. Discs are significantly more expensive than cartridges and for relatively simple, routine applications economic consideration often dictate the use of cartridges. Discs, on the other hand, offer specific format advantages that favor their use for some applications.

For large sample volumes containing suspended particles, discs are likely to function better than cartridges. Discs provide shorter sample processing times on account of their larger cross-sectional area and decreased pressure drop, allowing higher sample flow rates. This is important in environmental surveillance programs, such as the analysis of surface waters for trace contaminants, where large sample volumes are commonly employed to achieve adequate detection limits.

Discs have a large surface area per unit bed mass. This facilitates their use for passive sampling by immersing the disc in the sample as opposed to the conventional approach of passing the sample through the disc in a manner similar to filtration [37–39]. Passive sampling is convenient for both field and laboratory applications, but has been little explored so far. One reason is the slow equilibrium of the extraction process even for stirred solutions. The sorption of organic contaminants from water by an octadecylsiloxane-bonded silica disc using passive sampling under conditions that avoid sample depletion was used as an indirect measure of bioconcentration by biota [40,41]. The disc concentrates the more hydrophobic compounds from water in preference to hydrophilic compounds and is theorized to be a more realistic approach to assessing potential environmental consequences of organic contaminants in complex environmental systems. This is a relatively new approach to toxicity assessment and requires further work to establish a satisfactory relationship between the sorption properties of the disc and those of target aquatic species.

Because of the low packing density of typical cartridge devices, longer sorbent beds than are needed for extraction are used to compensate for reduced retention resulting from channeling. Increased bed mass results in increased

non-specific matrix adsorption and dirtier extracts. The use of smaller particles and the greater mechanical stability of discs minimize channeling, and the optimized use of bed mass results in a cleaner chemical background due to reduced matrix adsorption. For small sample sizes it is easier to miniaturize discs than cartridges, and several disc devices (e.g. microdiscs, pipette tips, etc.) that contain only a few milligrams of sorbent are available. Immobilized analytes on microdiscs facilitate integrated sample processing techniques such as in-vial desorption [42–44] and on-disc derivatization [42,44,45]. Solid-phase derivatization techniques for conventional sorbent cartridges are also described [46].

In-vial desorption reduces the amount of organic solvent used for recovery and eliminates tedious sample preparation steps compared to conventional sample processing methods. The in-vial desorption technique is an equilibrium-based approach to sample recovery. After extraction the dried disc is placed in an autosampler vial and covered with a few ml of solvent. Recovery depends on the selection of the solvent, temperature and equilibration time. An equilibration time of 4 h, or overnight, at room temperature is sufficient in many cases to provide acceptable recovery (>90%) and method precision as well as being compatible with automated sample analysis in gas and liquid chromatography. Addition of a derivatizing reagent to the solvent used for desorption allows derivatization and analyte recovery to be performed simultaneously in the same vial without removal of the disc. The vial can be sealed and heated to enhance the rate of reaction with the derivatizing reagent. Common reactions are trialkylsilylation and alkylation for gas chromatographic analysis. Ion-exchange discs catalyze the rate of alkylation of acid herbicides, surfactants and pesticides using a solution of an alkyl iodide as the derivatizing reagent. An example is shown in Fig. 12.4 [42].

The disc format supports other, if minor, applications at present, such as *in situ* detection using radioactivity counting [47], phosphorescence [48] and MALDI mass spectrometry [49,50]. The particle-embedded glass fiber discs can be inserted directly into a hole in a glass fiber sheet and developed in a conventional manner for thin-layer chromatography combining recovery and separation into a single step [51,52]. This approach is the basis of the Toxi-Lab® system for toxicological screening of biological fluids.

12.3 SORBENT TYPES AND THEIR APPLICATIONS

12.3.1 Inorganic oxide adsorbents

The most important inorganic oxide adsorbents for SPE are silica gel, alumina, Florisil and diatomaceous earth. Silica gels used for SPE have surface areas of about 300–800 m^2/g, pore sizes from 4–10 nm, and an apparent pH of 5.5–7.5 (measured indirectly as the pH of a 5% (m/m) sorbent suspension in water). Alumina used for SPE has a surface area of about 150 m^2/g and a pore size of 6 nm. Depending on processing conditions it is available as neutral (pH 7.5 ± 0.5), weakly acidic (pH 6.0 ± 0.5), acidic (pH 4.5 ± 0.5) and basic (pH 9.5 ± 0.5) forms.

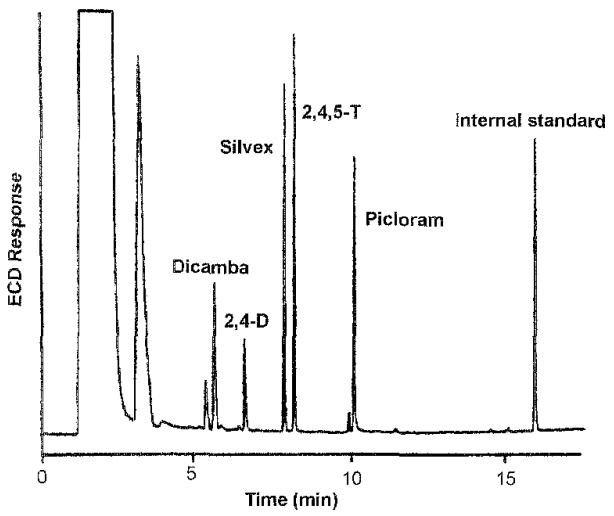


Fig. 12.4. Use of in-vial desorption and derivatization for the determination of chlorinated acid herbicides in an extract from a spiked (< 10 μg/l each) river water sample by gas chromatography. A 500-ml sample was processed through a 25-mm strong anion-exchange disc, the disc dried by suction, sealed in a autosampler vial with 1 ml of acetonitrile and 0.2 ml of methyl iodide and heated at 80°C for 1 h, and excess methyl iodide removed by evaporation. (From Ref. [42]; copyright Elsevier).

Florisil is a magnesium silicate with a surface area of about 250–300 m^2/g and an apparent pH of about 8.5. Diatomaceous earth is a flux-calcined silica with a low surface area used primarily as a filter aid and dispersant for solvent extraction by the matrix dispersion technique [53].

The general extraction mechanism and applications of the inorganic oxide adsorbents are summarized in Table 12.2 [1,4,5,54–57]. Adsorbent properties that increase retention are a larger surface area and a high activity. Adsorbent activity is usually controlled by the intentional addition of water to the dried adsorbent prior to use and by drying samples with anhydrous sodium sulfate, or a similar drying agent, prior to applying the sample to the adsorbent. A sodium sulfate cartridge connected in series to the adsorbent cartridge is a convenient approach for maintaining a constant adsorbent activity during sampling. Analyte properties that increase retention depend on the number and type of functional groups present. Hydrogen-bonding functional groups are strongly retained (e.g. sulfonic acid, carboxylic acid, phenol, hydroxyl, etc.), those with a significant dipole-character are retained to a lesser extent (nitro, ester, ketone, etc.) and polarizable functional groups (e.g. aromatic, alkene, etc.) are the least retained. Irreversible adsorption and catalytic breakdown of sensitive analytes can occur on all inorganic oxide adsorbents and is a source of low recovery for some analytes. Alumina and silica can function as selective ion-exchangers with buffered aqueous samples (see Table 12.2). Coating inorganic oxides with a complexing agent (e.g. silver nitrate, caffeine) or an acid or base (an acid can be used for the selective isolation of bases and *vice versa*) can be used to modify selectivity for the isolation of target compounds.

TABLE 12.2

General application of inorganic oxide adsorbents

Uses:

- Isolation of low and medium polarity analytes from non-aqueous solutions
- Isolation of cations (alumina and silica) and anions (alumina) from buffered aqueous solutions
- Matrix simplification by fractionation into groups containing a similar number and type of functional group

Examples:

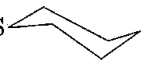
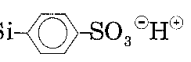
- Isolation of organochlorine pesticides and polychlorinated biphenyls from transformer oil, animal fats and oils, etc., using Florisil.
- Isolation of lipids by chromatography over silica gel using chloroform to elute simple lipids, acetone to elute glycolipids and methanol to elute phospholipids [54].
- Group fractionation of polycyclic aromatic compounds (hydrocarbons, N-containing, and OH-containing) in synthetic fuels over alumina using a step solvent gradient.
- Isolation of paraquat and diquat from high moisture crops in a pH 9 aqueous extract using silica gel [55,56]
- Mycotoxins in feeds using silica gel
- Pesticides in foods, feeds, and soil extracts [57]; alkaloids, pigments and flavor compounds from plants; sugars and caffeine in cola beverages, inorganic anions, and organic acids in aqueous solution using alumina; steroids and vitamins from creams and oil-based suspensions

12.3.2 Low-specificity sorbents

Low-specificity sorbents are widely used for the isolation of contaminants from aqueous solution. Because of the prevalence of water as a general solvent in environmental, biological and industrial chemistry these methods represent the most common applications of SPE. Typical sorbents include silica-based, chemically bonded materials, porous polymers [18,58–60] and graphitic carbon [8,61]. Silica-based, chemically bonded sorbents can be prepared with a wide range of bonding density, pore sizes and functional group types (Table 12.3). They are generally synthesized by reaction of monofunctional or trifunctional silanes with silica gel followed by endcapping in some cases. Trifunctional reagents result in sorbents with a polymeric bonded layer of higher carbon loading and greater pH stability. Silica gels of high surface area, 500–600 m^2/g, are generally used to prepare sorbents for the isolation of small molecules. Chemically bonded sorbents with high surface areas, long alkyl chains and high phase loading maximize retention of small molecules from aqueous solution while wide pore materials with a low phase loading and short alkyl chains are used to isolate macromolecules (Table 12.4) [1,4,5,62–69]. Chemically bonded sorbents with polar functional groups are used mainly to isolate analytes from organic solvents based on their selective interactions with analyte polar functional groups. They are less retentive than alkylsiloxane-bonded phases for aqueous samples. When the sampling process is not dominated by breakthrough

TABLE 12.3

Structures of silica-based chemically bonded sorbents

Type	Functional group	Structure
C18	Octadecyl	$-Si-C_{18}H_{27}$
C8	Octyl	$-Si-C_8H_{17}$
C2	Ethyl	$-Si-C_2H_5$
CH	Cyclohexyl	
PH	Phenyl	
CN	Cyanopropyl	$-Si-CH_2CH_2CH_2CN$
NH_2	Aminopropyl	$-Si-CH_2CH_2CH_2NH_2$
DIOL	2,3-Dihydroxypropoxypropyl	$-Si-CH_2CH_2CH_2OCH_2CH(OH)CH_2(OH)$
SAX	Trimethylaminopropyl (Quaternary amine)	$-Si-CH_2CH_2CH_2\overset{\oplus}{N}(CH_3)_3Cl^{\ominus}$
CBA	Carboxypropyl	$-Si-CH_2CH_2CH_2COOH$
SCX	Benzenesulfonic acid	
PRS	Propylsulfonic acid	$-Si-CH_2CH_2CH_2SO_3^{\ominus}H^{\oplus}$

volume considerations they can be used to minimize interference from matrix adsorption and to aid analyte recovery in a small solvent volume.

Silica-based, chemically bonded sorbents are the most widely used sorbents for SPE but are unsuitable for some applications. Breakthrough volumes for small, highly polar molecules in water are often too small for adequate quantification. Silica-based sorbents contain a low concentration of ionized silanol groups capable of retaining basic solutes by an ion-exchange mechanism [70]. Low recovery of these compounds can result from the inability of the eluting solvents to displace the analytes from the ion-exchange sites. Sometimes the addition of a competing base to the eluting solvent can solve this problem [4,70,71]. Silica-based sorbents are unstable at pH extremes ($2 > pH > 8$). Porous polymer and graphitic carbon sorbents offer a potential solution to these problems. They are stable throughout the full pH range and do not possess ionized silanol groups.

Modern porous polymer sorbents are generally copolymers of styrene and divinylbenzene processed to enhance their properties for SPE [19,58–60]. They

TABLE 12.4

Samples in aqueous solution

Samples in Aqueous Solution

- Isolation of neutral and ionic analytes from aqueous solution. Weak acids and bases by ion suppression. Strong acids and bases using ion-pair extraction (alternative to ion exchange)
- Retention increases with solute size and is reduced by polar interactions (particularly hydrogen-bonding) and ionization
- Polar bonded phases provide only weak retention and are not particularly useful unless elution of the analyte is a problem from non-polar sorbents

Examples

- Isolation of agricultural and industrial chemicals from surface waters using C18, Carbon or PS-DVB
- Isolation of polycyclic aromatic compounds from water by C18 and PS-DVB [62,63]
- Isolation of phenols from water by C18, Carbon and PS-DVB [64]
- Isolation of drugs from biofluids using C18, C8, PS-DVB, or CN [65]
- Isolation of macromolecules from biofluids and fermentation broth using C4
- Isolation of pesticides from biofluids using C18 and C8 [66,67]
- Isolation of antibiotics from biomatrices using C18 and C8 [68]
- Isolation of pigments and coloring materials from beverages and food extracts using C18
- Isolation of carbohydrates and nucleosides from biofluids using AMINO
- Isolation of proteins, peptides and surfactants using DIOL

Samples in Organic Solvents

- Retention depends on the type and number of functional groups. Solute size is less important.

CN	Strong dipole-type interactions and weak hydrogen-bond acidity
AMINO	Strong hydrogen-bond base, weak hydrogen-bond acid and weak dipole-type interactions.
DIOL	Strong hydrogen-bond acid, weak hydrogen-bond base and significant dipole-type interactions

Examples

- Isolation of polar pesticides from fats and oils
- Isolation of polycyclic aromatic compounds from fuel oils

Active ingredients from ointments and suppositories

are either similar in chemistry to porous polymers developed for HPLC and have moderate surface areas (< 600 m^2/g), or are biporous and highly crosslinked with surface areas from 700–1200 m^2/g. The larger surface areas of the highly crosslinked polymers results in higher retention. The highly strained porous structure is swollen to different extents by aqueous and organic solvents and is partially responsible for the favorable retention and elution properties of these materials [58]. Porous polymer particle-loaded membranes are selectively solvated by organic solvents, which are added to the sample as a processing aid (1% v/v). The solvated polymers exhibit significant selectivity differences resulting in significant compound-specific changes in breakthrough volumes (Table 12.5) [72]. The retention properties of poly(styrene-divinylbenzene) polymers can also be increased by light surface modification with polar functional groups, such as acetyl or sulfonate [59,73]. The polar substituents reduce the interfacial

TABLE 12.5

Breakthrough volumes (cm^3) of some organic compounds on porous polymer particle-loaded membrane discs with 1% (v/v) organic solvent in water

Compound	Processing solvent			
	Acetonitrile	Tetrahydrofuran	2-Propanol	Methanol
Anisole	575	300	1750	1300
Acetanilide	95	25	100	75
2-Phenylethanol	150	25	100	200
Heptan-1-ol	2700	300	2700	1000
Propyl propanoate	1400	200	1250	750

tension between the polymer surface and aqueous sample increasing contact between the analyte and polymeric sorbent. For sulfonated polymers increased retention of neutral polar compounds was strongly dependent on the concentration of sulfonate groups with an optimum concentration of 0.6 mmol sulfonate/g providing the largest increase in retention. The same goals are achieved by a macroporous poly(divinylbenzene-co-N-vinylpyrrolidone) polymer (Oasis™ HLB), which has a surface area of about 800 m^2/g [74]. The sulfonated polymers and vinylpyrrolidone copolymer have the added advantage of being fully water wettable. Consequently, a sorbent conditioning step prior to sample application is no longer required and samples can be fully recovered even if the sampling device runs dry during sample processing.

The modern forms of carbon used for SPE are graphitized carbon blacks and porous graphitic carbon [8,61,75–78]. Graphitized carbon blacks are (largely) non-porous with moderate surface areas of 100–210 m^2/g. Their surfaces are contaminated by oxygen complexes (hydroquinone, quinones, chromene) and benzpyrylium salts. Analyte retention occurs by a combination of adsorption and anion exchange. Porous graphitic carbon is a more refined material developed for HPLC and produced in a larger particle size for SPE. The retention mechanism is different to chemically bonded phases with high retention obtained for planar molecules containing several polar functional groups and for systems rich in polarizable electrons [8,77,78]. Recovery of adsorbed analytes from carbon sorbents using conventional solvents, such as methanol and acetonitrile, is often more difficult than for chemically bonded sorbents. Stronger (less polar) binary solvent mixtures are often more efficient and desorption by backflushing is recommended for well-retained compounds.

Low-specificity sorbents are commonly used for multiresidue analysis of pesticides, herbicides, etc., and their decomposition products in environmental and food samples [30,79–82]. These applications are relatively recent but represent a natural extension of SPE methods for use in surveillance programs. No single sorbent is considered ideal for these applications at present, sustaining interest in the development of new sorbents with optimized retention properties

to meet regulatory requirements. Ion-pair extraction is a useful approach for isolating ionized compounds by low-specificity sorbents but is not widely used at present [83].

12.3.3 Compound and class-specific sorbents

Various selective sorbents based on ion exchange, bioaffinity, molecular recognition, and restricted access materials are used to supplement the sorbents discussed above. Ion exchange is used to isolate inorganic and organic ions (usually) from aqueous solution with sorbents containing fixed ionic sites of opposite charge to the analytes of interest (Table 12.6) [1,4,5,84]. Ion-exchange sorbents are usually classified as weak or strong depending on the identity of the ionic group and whether its charge is independent of the sample pH (strong ion exchanger) or can be manipulated by changing pH (weak ion exchanger). Some examples of typical silica-based, ion-exchange sorbents are indicated in Table 12.3. Porous polymer, ion-exchange resins are also commonly used in SPE and have a higher exchange capacity and a wider pH operating range than silica-based materials. For many applications silica-based and porous polymer ion-exchange materials with the same immobilized ionic groups can be used interchangeably, although because of non-specific adsorption of matrix components the chemical background of the extracts might be different. Ion-exchange materials are particularly attractive for the isolation of ions since the neutral molecules, which may interfere in the final chromatographic analysis, are easily rinsed from the ion exchanger without affecting the recovery of the ions.

In recent years disc-based SPE has become a popular technique for sample clean-up in ion chromatography and capillary electrophoresis [85,86]. Interfering ions, often at a relatively high concentration, can mask, broaden or change the migration time of the ions of interest. Removal of interfering ions is

TABLE 12.6

General applications of ion-exchange sorbents

- In general strong ion exchanges are used to isolate weak acid/bases of opposite charge and weak ion exchanges strong acid/bases
- Retention selectivity can be adjusted by manipulating the sample pH and ionic strength
- Choice of competing ion, its concentration and eluent pH controls selectivity for matrix simplification and elution
- Isolation of macromolecules in an active form may require special non-denaturing sorbents based on cellulose, agarose or dextran

Examples

- Isolation of carboxylic, sulfonic and phosphoric acids, phenols, amines and inorganic ions from water
- Isolation of amino acids, organic acids, nucleosides and nucleotides from biofluids
- Isolation of organic acids and bases from coal-derived and synthetic fuels

Isolation of organic acids, phenols and amines from wine, fruit juices and food extracts

- Isolation of acid herbicides from water [84]

performed by disc extraction using a sulfonic acid-functionalized resin in the hydrogen, silver or barium forms. The hydrogen form is used to remove hydroxide and carbonate ions from samples by neutralization. The silver form is useful for the removal of excess halide ions from samples by formation of insoluble silver halide salts that precipitate in the disk matrix. The barium form is used to remove sulfate from samples through the formation of insoluble barium sulfate.

12.3.3.1 Mixed-mode and multimode extractions

Mixed-mode sorbents containing co-bonded ion-exchange and alkyl groups in either cartridge or disc format are popular in clinical and pharmaceutical laboratories, where they are used for the isolation of drugs with ionizable functional groups and their metabolites from biological fluids [4,5]. Standard protocols using mixed-mode sorbents have been developed for the isolation of most drugs of abuse (e.g., amphetamines, barbiturates, cocaine, opiates, etc.) and for classifying extracts into acid/neutral and basic drugs for systematic toxicological analysis [86–90]. Drugs are initially retained by a reversed-phase mechanism and excess salts and polar neutral molecules (e.g. urea) are washed from the sorbent. An acid rinse is used to protonate bases increasing their retention by an ion-exchange mechanism. Elution with an organic solvent selectively isolates the neutral and acid fraction. Subsequent elution with an aqueous-organic eluent containing a base elutes the basic drugs. The strong retention and the use of efficient rinse solvents result in cleaner extracts compared with single mode sorbents, suitable for screening by thin-layer chromatography and confirmation by gas chromatography–mass spectrometry or liquid chromatography–mass spectrometry.

Discs or cartridges with different sorbent chemistries can be staked on top of each other and analytes recovered with a single elution or as fractions of different chemistries after physically separating the discs or cartridges [3,4, 43,91]. This approach is useful for complex samples containing analytes that differ significantly in polarity or ionization, for optimizing the breakthrough volume of analytes, and for reducing interference in the chromatogram by the selective sorption of the matrix by the first SPE device and the analytes by the other. Because of ease of stacking discs with minimal dead volume this approach is more common using discs than cartridges, but in both cases general use is less than might be anticipated given the simplicity of the procedure. Stacked discs of a reversed phase and cation exchange type are a suitable alternative to mixed-mode sorbents for isolating drugs and their metabolites from biological fluids. Cation-exchange discs have been used to remove humic and fulvic acids from surface waters and soil extracts with retention of phenylurea and triazine herbicides on a octadecylsiloxane-bonded silica disc [92]. A combination of a octadecylsiloxane-bonded silica disc and a carbon disc was used to isolate N-nitrosodimethylamine from water in which the octadecylsiloxane-bonded silica disc was used to sorb matrix components and set aside before elution of the analyte from the carbon disc [93].

12.3.3.2 Restricted access materials

Restricted access sorbents were initially developed for the isolation of low-molecular-mass drugs from biological fluids with minimum sample pretreatment [94–100]. Recent applications include the isolation of acidic herbicides from surface waters burdened by humic and fulvic acids [101]. Restricted access materials work by preventing access of macromolecules (proteins) to those regions of the sorbent where analyte retention occurs (Fig. 12.5). Restricted access to the retentive part of the sorbent is provided by either a physical diffusion barrier, such as a pore diameter in internal surface reversed-phase sorbents, or by a chemical diffusion barrier, such as a polymer network at the outer surface of the particle in semi-permeable surface materials. In addition, the outer surface of the particles must be non-adsorptive and matrix-compatible. Restricted access sorbents are commonly used for automated on-line sample processing in liquid chromatography. In this case a short precolumn (0.5 to 3.0 cm) packed with the restricted access material is interfaced to a separation column by a multiport switching valve (see Section 12.6.1). The biological fluid is injected directly onto the precolumn, which retains the analytes of interest. Interfering macromolecules (proteins) pass through the precolumn unretained and are flushed to waste. The analytes retained on the precolumn are eluted on-line to the separation column and detected. Simultaneously the precolumn is reconditioned (or exchanged) before processing the next sample. An important consideration for automated sample processing is the ability of the restricted

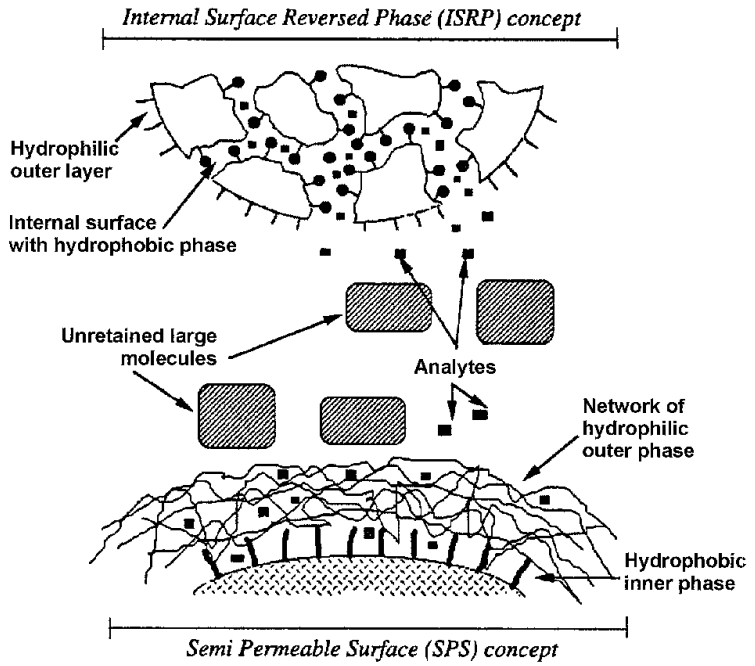


Fig. 12.5. Schematic representation of the separation mechanism of restricted access materials. (From Ref. [101]; copyright Elsevier).

access material to repeatedly extract the analyte without change in properties or accumulation of sample matrix components.

12.3.3.3 Surface-bound macrocyclic ligands

Silica and porous polymer sorbents with surface-bound macrocyclic ligands can be used for the selective isolation and concentration of alkali–metal, alkaline–earth–metal, post-transition metal ions, and some anions from aqueous solution and complex sample matrices of high ionic strength (e.g. seawater) or extreme pH [47,102–105]. It is particularly useful for those conditions where ion exchange is likely to be ineffective because of a lack of retention or selectivity. Metal complexation by macrocyclic ligands is generally tolerant of extreme pH and ionic strength. The stability of the host–guest complex, and therefore the selectivity of the isolation process, depends on matching the metal ion radius to the size of the macrocycle cavity and the selection of the donor atom types. The metal ion is sorbed in the cavity of the macrocycle during the sample application phase and later released by elution with a solution of a complexing agent with a higher binding constant for the metal. Both processes provide a high level of specificity allowing sorbents to be developed for specific applications. One significant application is the use of RAD discs in the nuclear industry for analyzing groundwater and nuclear fuel storage-pool water for trace quantities of radionuclides such as strontium, radium, technetium, etc. [47,105].

12.3.3.4 Surface-bound phenylboronic acids

Phenylboronic acid groups covalently attached to silica or porous polymer supports are used for the selective isolation of compounds containing vicinal diol groups (e.g., nucleosides, catecholamines, steroids, drugs, etc.), α-hydroxy acids, 1,2-aminoalcohols, 1,2-diketones, 1,3-diols, etc., by formation of 5- or 6-membered cyclic boronate complexes [4,5,106,107]. The selectivity of the retention mechanism depends on the stability of the covalent complex, which is largely governed by ring size, functional group type and pH. Complex formation requires a basic pH. This is achieved by activating the sorbent with an alkaline buffer at pH 10–12 followed by a rinse and application of the sample at (usually) a pH of 8.0–8.5. Zwitterionic buffers (Good's buffers) are typically used for pH control. After formation of the covalent complexes, the cartridge is washed with alkaline aqueous-organic solvent mixtures to remove non-specifically adsorbed matrix components. The analytes are recovered by elution with an acidic aqueous-organic solvent mixture (pH < 5), which decomposes the covalent complexes releasing the bound analytes.

12.3.3.5 Immunosorbents

Immunosorbents (or immunoaffinity sorbents) have been used for a long time for sample pretreatment in medicine, biology and food science but more general applications, such as to environmental samples, are relatively recent [108–113]. In part this is due to the difficulty of making antibodies selective to small

molecules as well as a lack of familiarity among analytical chemists with the procedures used to make specific antibodies. The interest in immunosorbent extraction results from its high selectivity, which allows extraction, concentration and clean-up from complex matrices in a single step and from large sample volumes when required. The high degree of molecular selectivity is due to the specificity of the antibody-antigen (analyte) spatial fitting and interactions. Cross-reactivity, considered a disadvantage in the development of single compound schemes, is advantageous for multiresidue methods. Class-specific immunosorbents for mycotoxins, phenylurea herbicides, and polycyclic aromatic hydrocarbons, for example, are commercially available. Manufactured immunosorbents have been available for a relatively short time and the range of products remains narrow. Most applications continue to emanate from a few research groups. It is clear, however, that immunosorbents have a good potential for use with difficult sample matrices. Products based on immunochemistry have been well received in other areas of analytical chemistry [114].

Immunosorbents are prepared by covalently bonding a suitable antibody to an appropriate sorbent. These sorbents are used in disposable cartridges or as short precolumns in coupled-column liquid chromatography, which has received the most attention in research laboratories. For the latter application it is important that the sorbent is pressure stable and the precolumn reusable for many samples. Coupled-column SPE-GC is not as advanced [115] as SPE-LC but has good potential for future growth.

The breakthrough volume of an immunosorbent cartridge or precolumn depends on the total number of accessible binding sites and on the analyte–antibody binding constant. It is desirable to minimize non-specific analyte and matrix interactions with the support. As a consequence there is no universal antibody support suitable for all applications. The analyte capacity of immunosorbents is generally low, but since these materials are usually used for trace analysis and their selectivity for the target analytes is high, this is not normally a problem. The strong analyte–antibody binding facilitates rinsing the sorbent with solvents to eliminate matrix interference. Finding conditions that release the analytes in a small elution volume can be problematic, particularly if the immunosorbent is to be reused. Aqueous organic solvent mixtures are commonly used for elution but may denature the antibodies; alternatives include the use of competitive binding (displacing) agents, change in pH or temperature variation. It is clear that the selection of sample processing conditions with immunosorbents requires some adaptation of conventional SPE procedures, but when fully optimized they are no more difficult to use than conventional sorbents.

12.3.3.6 Molecularly imprinted polymers

Molecularly imprinted polymers are sometimes referred to as plastic antibodies and are used in SPE as synthetic analogs of immunosorbents [116–123]. Molecular imprinting is a technique used for preparing polymers with synthetic recognition sites having a predetermined selectivity for a specified analyte (or

group of similar analytes). The imprint is obtained by the polymerization of functional and cross-linking monomers in the presence of a template molecule (the analyte). The template–monomer system is chosen such that in solution the imprint molecule complexes one or several of the functional monomers, which then become spatially fixed in a solid polymer by the polymerization reaction. The resultant imprints possess a steric (size and shape) and chemical (spatial arrangement of complementary functional groups) memory for the template molecule. Removal of the template from the polymer matrix creates vacant recognition sites that enable the polymer to selectively rebind the imprint molecule from a mixture of closely related compounds. In some cases the binding affinity and selectivity for template–polymer binding approach those demonstrated by antibody–antigen complexes.

For the time being, it is difficult to predetermine the experimental conditions for successful imprinting of target analytes. In the case of non-covalent binding, the most common synthetic method (other approaches include reversible covalent interactions and metal ion mediated interactions), the key to a successful imprint polymer is the formation of a stable complex between monomer and template molecules in the pre-polymerization mixture. Maximal efficiency of imprint formation occurs when the solvent (porogen) has sufficient solvating power to adequately solubilize the reaction mixture without interfering with the formation of template–monomer interactions. In current practice apolar organic solvents for synthesis of the imprint polymer are generally used, which are usually different to solvents used for sample application. Shrinking or swelling of the polymers in different solvents during sample processing and recovery can have a profound effect on the affinity of the polymer for the template molecule [117,122–124]. To date, block polymerization using UV radiation or thermal initiation to create a macroporous polymer, followed by grinding and particle sizing, has been the technique most often used for the preparation of polymer particles for cartridge SPE. The synthesis of the imprint occurs in the presence of a large amount of the template molecule, which can be difficult to quantitatively extract from the polymer reducing its binding capacity, but more seriously, residual template molecules may leak into sample extracts disqualifying analyte quantification, particularly at trace concentrations. One solution to this problem is to use a template molecule similar to the analyte for the imprint process together with an analytical method that is able to separate the template and analyte for the determination step. Only a few practical applications of molecularly imprinted polymers for SPE have been demonstrated so far [117,119], most of which are for the isolation of drugs from biological fluids, but there is considerable promise for this technology. Methods for controlling non-specific adsorption and polymer morphology and stability are required, but seam well within the bounds of polymer technology used to prepare sorbents for HPLC. Molecularly imprinted polymers should be easier and cheaper to produce in chemical laboratories than antibodies while, at least in theory, they should be capable of similar specificity.

12.4 THEORY OF SOLID-PHASE EXTRACTION

Most of the parameters that describe the sequence of processing steps in SPE are amenable to measurement by liquid chromatography or estimation using theoretical principles derived from the theory of liquid chromatography [4,6,125–127]. Analyte concentrations are generally low and the volume of sample that can be processed, and therefore the amount of analyte isolated, is determined by the breakthrough volume of the sampling device. The volume and type of rinse solvent for matrix simplification is determined by the type and amount of sorbent required for the isolation step. Here, the need is to identify the volume of the strongest solvent that eliminates matrix components from the sorbent without analyte loss. Optimum conditions can be established by selecting eluting conditions that preserve a certain minimum value for the retention factor of the least retained analyte. To accomplish a significant concentration of the analytes with minimal further sample manipulation it is desirable to recover the analytes in a small solvent volume. Generally the minimum elution volume that can be safely employed is about 2–3 times the hold-up volume for the sampling device. This corresponds to a retention factor less than 2 for the most retained analyte. In addition, the sorption mechanisms for sample application occur according to the characteristics of frontal analysis and the rinse and desorption step by elution. Typical SPE devices contain short sorbent beds and cannot be expected to provide a high plate number. The possibility that retention may be influenced by kinetic properties has also to be taken into consideration.

12.4.1 Breakthrough volumes

The breakthrough volume for a particular analyte and sampling device is determined by its breakthrough curve (Fig. 12.6). In the initial sampling phase the analytes are quantitatively retained by the sorbent up to the point that the sample volume exceeds the retention capacity of the sorbent. Further sample passing through the sorbent bed is not quantitatively retained by the sorbent and eventually the analyte concentration entering and exiting the sampling device become identical. The position on the curve at which some arbitrary amount of sample is detected at the outlet of the sampling device, typically 1%,

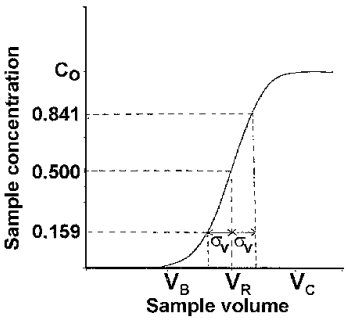


Fig. 12.6. Typical representation of a breakthrough curve. V_B is the breakthrough volume, V_R the chromatographic elution volume, V_C the sample volume corresponding to the isolation of the maximum amount of analyte, C_0 the concentration of analyte in the sample, and σ_V the standard deviation of the derivative curve for the plot. (From Ref. [127]; copyright Elsevier).

5% or 10% is arbitrary defined as the breakthrough volume (V_B). The chosen level reflects the difficulty of experimentally determining small changes in the concentration of the analytes at the outlet of the sampling device. For the purpose of modeling the breakthrough process a value of 1% is generally chosen in keeping with the desire to define a maximum sample volume that can be processed with minimal (acceptable) analyte loss. A second point on the breakthrough curve, V_C, corresponds to the sample volume at which the retention capacity of the sorbent is saturated and the concentration of analyte exiting the sampling device is the same (actually 100% used to define the breakthrough volume) as that entering the sampling device. It corresponds to the volume of sample that will result in the isolation of the maximum amount of analyte but with a lower overall recovery, because a fraction of the sample is lost during the sorption process. The point of inflection for the breakthrough curve corresponds to the chromatographic retention volume, V_R, provided that the plate number for the sampling device is not too small [128].

In general, there are two common causes of premature breakthrough in frontal chromatography. The retention capacity of the sorbent bed is overloaded due to a high concentration of either analyte or sorbed matrix components. Or, the sorbent bed may fail to adequately retain the analytes due to the provision of an insufficient plate number for retention volumes to be independent of the plate number for the sampling device.

12.4.1.1 Determination of breakthrough volumes

The most straightforward method for determining breakthrough volumes is the direct method using either on-line or off-line detection [16,129]. This method is particularly convenient for precolumn devices used in on-line SPE-LC. A solution containing a low but detectable concentration of analyte is pumped at a constant flow rate through the precolumn, which is connected directly to the detector. The detector response for the analyte at the exit of the precolumn is similar to Fig. 12.6. Since the breakthrough volume may be flow rate dependent the flow rate used to record the breakthrough curve should be similar to the flow rate used for sample application.

For standard cartridge and disc devices off-line sample processing is commonly used. Samples are processed in aliquots in the same way as regular samples with an off-line detection method used to determine the analyte concentration in the extracts recovered from each sample [72,130–132]. Each aliquot contains the same amount of analyte but in a different sample volume. Initially, an approximate value of the breakthrough volume is established by using decade changes in aliquot volumes, followed by a more systematic experimental design. For compounds with an estimated breakthrough volume between 0 and 50 ml, measurements are made at 2.5 ml volume increments, between 50 and 100 ml at 5 ml increments, 100 and 1000 ml at 10 ml increments, and greater 1000 ml at 100 ml increments. Plotting the observed recovery for the complete sampling process against the corresponding aliquot volumes generates a breakthrough

curve from which the breakthrough volume is estimated from the best-fit line through the experimental data. A similar approach has been used to determine the breakthrough volume of precolumn traps in on-line SPE-LC [133–135].

12.4.1.2 Methods for estimating breakthrough volumes

The determination of breakthrough volumes is time consuming and identifying the breakthrough point on the breakthrough curve somewhat subjective. Consequently, methods that enable breakthrough volumes to be estimated from solute properties or calculated from models that require a minimal number of experimental measurements are particularly attractive. The octanol–water distribution constant ($\log K_{OW}$) is a widely used parameter for estimating compound hydrophobicity and as a general compound descriptor for modeling numerous environmental and biological properties [136]. Hennion et al. [125] investigated the use of $\log K_{OW}$ to estimate $\log k_W$ (sorbent–water retention factor) for the purpose of modeling SPE breakthrough volumes but concluded that it was of limited value. These results were confirmed by Poole et al. [136] and explained in a theoretical context.

12.4.1.3 Models for predicting breakthrough volumes

From the general theory of frontal chromatography it is possible to derive a relationship between the breakthrough volume and the sorption properties of SPE devices [16,76,125–127,137–141]. At the 1% breakthrough level the breakthrough volume, V_B, is related to the retention volume, V_R, through Eq. (12.1)

$$V_B = V_R - 2.3\sigma_V \tag{12.1}$$

where σ_V is the standard deviation depending on the axial dispersion of the analyte along the sorbent bed (see Fig. 12.6) and is evaluated through Eq. (12.2)

$$\sigma_V = V_M(1 + k)/\sqrt{N} \tag{12.2}$$

where V_M is the interparticle volume of the sorbent bed (hold-up volume), k the retention factor, and N the plate number for the sorbent bed calculated by Eq. (12.3)

$$N = V_R(V_R - \sigma_V)/\sigma_V^2 \tag{12.3}$$

In principle it should be possible to calculate V_B from Eqs. (12.1) to (12.3) by determining V_M and N for the sampling device and measuring V_R (or k) for the analytes of interest. N and V_M are easily determined for precolumn cartridges in coupled SPE-LC since the on-line arrangement allows direct recording of the breakthrough curves. There is no convenient way to make measurements of these parameters for off-line sampling conditions, and therefore estimates must be made from results obtained for the SPE device by HPLC [127,128,142]. Equations (12.1) to (12.3) are derived assuming that the conditions of linear chromatography apply and the plate number for the sorbent trap is reasonably

TABLE 12.7

Coefficients for the Lovkvist and Jonsson model, Eq. (12.4), for sorbent beds with a low plate number

Breakthrough level (%)	Coefficients		
	a_o	a_1	a_2
0.1	0.998	29.12	57.54
0.5	0.990	17.92	26.74
1.0	0.980	13.59	17.60
5.0	0.903	5.36	4.60
10.0	0.810	2.88	1.94

large. For sorbent beds with low plate numbers the above equations can result in a poor estimate of breakthrough volumes.

Lovkist and Jonsson [128] proposed a model described by Eq. (12.4) to characterize the sampling properties of sorbent beds with a small plate number that has been adopted by other groups [127–131,142–144] to calculate breakthrough volumes under SPE conditions. The coefficients a_o, a_1, a_2 are characteristic of the breakthrough level and are summarized in Table 12.7 [128]

$$V_B = (a_o + a_1/N + a_2/N^2)^{-1/2}(1 + k)V_M \quad (12.4)$$

The calculation of the breakthrough volume requires the determination of N and V_M for the sampling device and either a measurement or estimation of the retention factor. The influence of these parameters with respect to the performance of solid-phase extraction devices can now be discussed. The main requirements are the optimization of size (V_M and N), kinetic properties (N, particle size, flow rate) and retention (N, k and V_M).

12.4.2 Kinetic optimization of cartridge and disc devices

For off-line or on-line sampling the sorbent bed cannot be too large because it is desirable to recover the analytes in a small solvent volume. In addition, in off-line sampling the pressure drop available to transport the sample through the sampling device at a practical velocity is limited. The pressure drop per unit length of sorbent bed is given by Eq. (12.5) [127]

$$\Delta P/L = u\eta\phi/d_P^2 \quad (12.5)$$

where ΔP is the pressure drop across a sorbent bed of length L, u the linear velocity of the sample solution through the sorbent bed, η the viscosity of the sample solution, ϕ the flow resistance parameter for the sorbent bed (typically 10^3 for cartridges and particle-loaded membranes [126]) and d_P the average

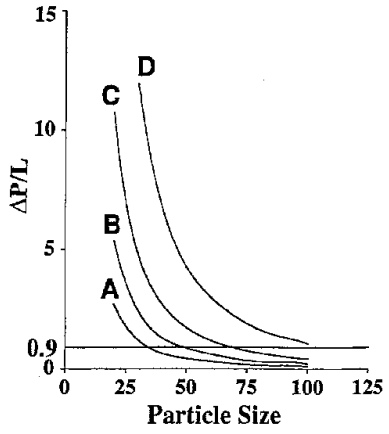
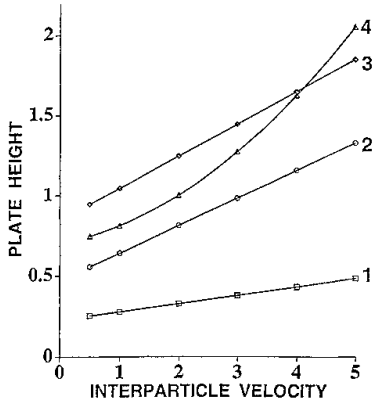
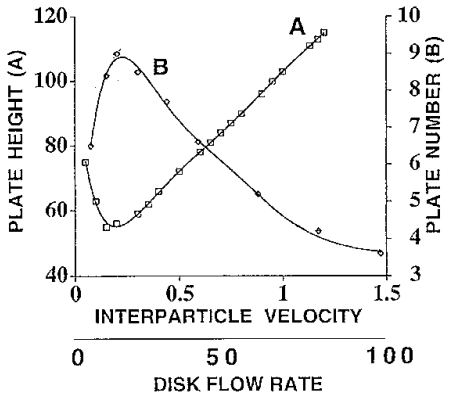


Fig. 12.7. Plot of ($\Delta P/L$) against the average particle size (d_P) for different sample processing rates (u). A = 0.11 mm/s, B = 0.22 mm/s, C = 0.43 mm/s and D = 1.08 mm/s. The horizontal line represents the cut off for a pressure drop of 0.9 atm for a 1 cm bed length. (From Ref. [127]; copyright Elsevier).

particle diameter for the sorbent. For off-line sampling with cartridge devices the available pressure drop is limited to about 0.9 atm. In practice this means that sorbent beds are restricted to particle diameters greater than about 40 μm with larger particles providing a wider range of sample flow rates (Fig. 12.7) [127]. For coupled-column systems high-pressure pumps are used for sample application and pressure is no longer the factor that dictates the sorbent particle diameter employed. These columns are also generally short resulting in conditions favorable for fast sample processing. For off-line sampling the easiest solution to increase sample-processing rates is to increase the diameter of the sampling device at a constant bed height, since the flow rate is proportional to the diameter squared. This is conveniently achieved using particle-loaded membranes and particle-embedded glass fiber discs.

Sorbent cartridges with short beds packed with coarse particles, or for that matter, very short beds packed with fine particles, cannot be expected to provide large plate numbers. A plot of the plate height against the interparticle velocity for some typical SPE sorbents is shown in Fig. 12.8 [126]. There is no observed minimum in the plots over the interparticle velocity range of 0.5–5.0 mm/s (equivalent to a flow rate of about 3–30 ml/min through a 1-cm diameter cartridge). The main contribution to the plate height arises from flow anisotropy in the packed bed and resistance to mass transfer. The sorbents in Fig. 12.8 provide from 5–40 plates per centimeter of bed length with considerable variation for the different sorbent types. A cartridge with a 1-cm diameter operated at a sample flow rate of 1 ml/min cannot be expected to provide more than about 20 plates per centimeter of bed length. At higher sample flow rates only lower plate numbers are expected. For cartridges with a lower packing density than shown in Fig. 12.8, a realistic scenario based on measurements of average cartridge packing density [126] even smaller plate numbers are to be expected.





Left: Fig. 12.8. Plot of the plate height (mm) against mobile phase interparticle velocity (mm/s) for silica-based cartridge sorbents. The test compound was anthracene and the mobile phase methanol–water (80:20 v/v). 1 = Octadecylsiloxane-bonded sorbent (light loading); 2 = spacer bonded propanediol sorbent; 3 = cyanopropylsiloxane-bonded sorbent; 4 = butylsiloxane-bonded sorbent. (From Ref. [126]; copyright Elsevier).

Fig. 12.9. Variation of the efficiency of a particle-loaded membrane as a function of the mobile phase velocity. (A) Plot of the observed plate height (μm) as a function of the interparticle mobile phase velocity, and (B) the transformation of (A) to indicate the plate number (N) as a function of the sample flow-rate through a 0.5 mm disk with a 38 mm-diameter active sampling area (From Ref. [126]; copyright Elsevier).

The kinetic properties of particle-loaded membranes are different to those of cartridges (Fig. 12.9) [126,145]. A minimum value for the plate height, corresponding to about seven particle diameters, is observed at an optimum interparticle velocity of about 0.19 mm/s. Over the typical flow rate range for a 47-mm-diameter disc with a 38-mm active sampling area, 10–100 ml/min, the particle-loaded membrane will provide about 4–9 theoretical plates, with the highest value in the region of the optimum flow rate (about 13 ml/min).

The influence of low plate numbers on breakthrough curves for SPE devices can be calculated using Eq. (12.4). Breakthrough curves are given in Fig. 12.10 for a cartridge with a hold-up volume of 0.42 ml, a solute retention factor of 100, providing 5, 20 and 100 plates per cm bed length. Increasing plate numbers results in a sharper front boundary and a larger breakthrough volume (23 ml for $N = 5$, 34 ml for $N = 20$ and 40 ml for $N = 100$). Larger plate numbers are desirable for optimal sample retention but small values of N are capable of providing useful breakthrough volumes.

For recovery of analytes by elution it is necessary to consider the shape of the front as well as the retention capacity of the sorbent [76]. The required elution volume for 99% analyte recovery, V_E, on a cartridge with a low plate number is given by Eq. (12.6)

$$V_E = V_M[1 + k][1 + (2.3/\sqrt{N})] \quad (12.6)$$

The only practical way to minimize the volume of eluting solvent is to use a small sorbent bed (minimize V_M) and a strong solvent ($k < 3$ and ideally 0). Cartridges

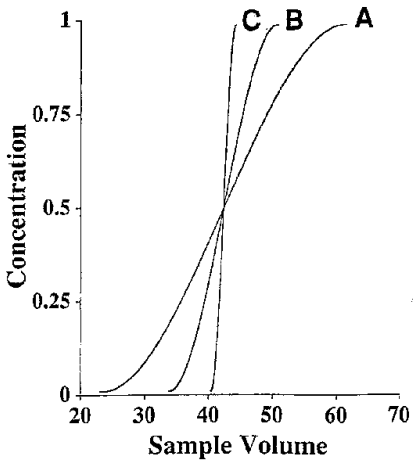


Fig. 12.10. Plot of the breakthrough curves for a sampling device with a hold-up volume of 0.42 ml, retention factor 100 and (A) 5, (B) 20, and (C) 100 plates per cm of bed length. (From Ref. [127]; copyright Elsevier).

with relatively large values of N provide sharper desorption front profiles and require a smaller elution volume to quantitatively recover the analyte from the cartridge.

12.4.3 Retention

It is convenient to rearrange Eq. (12.4) into the general expression

$$\log V = \log QV_M + \log (1 + k_S) \tag{12.7}$$

where V is either the breakthrough volume, volume of rinse solvent or elution volume, Q the contribution of kinetic factors to retention resulting from the small plate number, V_M the hold-up volume for the sorbent bed, and k_S the retention factor for the analyte with the sample solvent, rinse solvent or elution solvent as mobile phase. For a limited range of flow rates and a specified sampling device, the product QV_M is approximately constant. Given the typical numerical values for QV_M it is easy for $\log (1 + k_S)$ to become the dominant term in Eq. (12.7) and the most important factor determining the breakthrough volume. To optimize the breakthrough volume requires the selection of sample processing conditions that provide large retention factors. Selection of a rinse solvent requires identification of experimental conditions that preserve a sufficiently large retention factor to avoid loss of analyte during the rinse step. The selection of the elution conditions requires identification of experimental conditions that minimize the retention factor. Methods that allow the measurement or estimation of retention factors for relevant sampling conditions are then of prime importance for method development in SPE.

12.4.3.1 Experimental determination of retention factors

There are two general approaches for determining retention factors on sorbents used for SPE. The equilibrium method uses a fixed volume of sample solution with a known concentration of analyte that is continuously circulated by a mechanical pump through the sampling device and returned to the sample solution reservoir until a steady state is reached [36,140,141]. On-line monitoring of the analyte solution exiting the sampling device is a convenient method to establish the time required to reach a steady state. The amount of analyte retained by the sorbent is then determined by elution with a small volume of strong solvent using any suitable method for quantification. Provided that the sorption isotherm is linear the retention factor can be calculated from the volume and concentration of the analyte in the sample solution, the hold-up volume for the sorbent bed, and the amount of analyte taken up by the sorbent under steady-state conditions.

The alternative method of determining the retention factor is by direct measurement of retention in a typical chromatographic experiment using columns of different lengths packed with the SPE sorbent [139,142,146–150] or forced-flow planar chromatography [28,145,151] for discs. Compounds with retention factors up to about ten thousand can be determined in this way. Compounds with a retention factor greater than ten thousand are more than adequately retained for most likely sampling conditions and an accurate determination of their retention factors is rarely required.

12.4.3.2 Methods for estimating retention factors

The general approach for estimating retention factors with sample solutions containing predominantly water is by extrapolation from retention factors determined at more convenient mobile phase compositions providing shorter separation times. Extrapolations are usually based on either linear, Eq. (12.8), or quadratic, Eq. (12.9), models

$$\log k = \log k_W - S\phi_S \tag{12.8}$$

$$\log k = \log k_W + S_1\phi_S + S_2\phi_S^2 \tag{12.9}$$

where k_W is the equation intercept (assumed to be equivalent to the retention factor for the analyte with water as the mobile phase), ϕ_S the volume fraction of organic solvent in a binary mixture of water and organic solvent, and the S coefficients are regression constants obtained by fitting the experimental data to the models. The validity of Eqs. (12.8) and (12.9) is debated elsewhere [125–127, 136,152,153]. Here we simply note that either equation may function adequately for a limited range of aqueous organic solvent mixtures of intermediate composition but both equations are unreliable when data for the region of low organic solvent composition of interest for estimating breakthrough volumes in SPE are included [125–127,136]. Some typical results are summarized in Table 12.8

TABLE 12.8

Comparison of experimental and extrapolated values for log k_W for an octadecylsiloxane-bonded silica sorbent

Compound	Log k_W		
	Linear Eq. (12.8)[1]	Quadratic Eq. (12.9)[2]	Experimental
Methanol-Water			
2-Phenylethanol	2.00	2.36	2.45
4-Chlorophenol	2.52	2.50	2.73
4-Nitrobenzyl alcohol	1.58	2.15	2.40
Acetanilide	1.60	2.19	2.52
Acetophenone	2.26	2.81	3.01
Benzaldehyde	1.87	2.43	2.56
Hexan-2-one	1.91	2.49	2.62
Acetonitrile-Water			
2-Phenylethanol	1.27	2.05	2.45
4-Chlorophenol	1.81	2.35	2.73
4-Nitrobenzyl alcohol	1.21	1.89	2.40
Acetanilide	1.03	1.92	2.52
Acetophenone	1.71	2.33	3.01
Benzaldehyde	1.66	1.94	2.56
Hexan-2-one	1.74	2.31	2.62

[1]From 40–70% (v/v) methanol or acetonitrile.
[2]For the full data range from 1–100 % (v/v) methanol or acetonitrile.

[136]. Because extrapolation methods provide unreliable estimates of log k_W with large errors likely in some cases, extrapolation methods cannot be used to estimate log k_W with any reasonable level of confidence for estimating breakthrough volumes by Eq. (12.7).

12.4.3.3 Solvation parameter model

The appropriate form of the solvation parameter model for SPE with liquid samples is given by Eq. (12.10) [72,126,127,132,151,153].

$$\log SP = c + mV_X + rR_2 + \pi_2^H + a\sum\alpha_2^H + b\sum\beta_2^H \qquad (12.10)$$

The model equation is made up of product terms representing solute properties (descriptors) and system properties characteristic of the sampling system. Each product term represents the contribution of a defined intermolecular interaction to the correlated solute property (SP), in this case either the retention factor (log k) or breakthrough, rinse and elution volumes (log V) when log QV_M in Eq. (12.7) is approximately constant.

The solute descriptors in Eq. (12.10) are McGowan's characteristic volume V_X, the excess molar refraction R_2, the solute's dipolarity/polarizability π_2^H, and

the solute's effective hydrogen-bond acidity and hydrogen-bond basicity, $\sum \alpha_2^H$ and $\sum \beta_2^H$, respectively. The solute's characteristic volume and excess molar refraction are additive properties that can be calculated for any analyte whose structure is known [154,155]. The solute dipolarity/polarizability and hydrogen-bond acidity and basicity parameters can be obtained experimentally from gas–liquid chromatographic data or water-solvent distribution constants [156]. For some solutes such as anilines, pyridines, and sulfoxides, which exhibit variable hydrogen-bond basicity in aqueous and non-aqueous solutions, the $\sum \beta_2^H$ descriptor is replaced by $\sum \beta_2^0$ for the extraction of samples from aqueous solution. The $\sum \beta_2^H$ descriptor is retained for extraction of analytes from organic solvents. Solute descriptors are available for about 4000 compounds with others available through parameter estimates for compounds of known structure [157].

The system constants in Eq. (12.10) are defined by their complementary interactions with the solute descriptors. The *r* constant determines the difference in capacity of the solvated sorbent and sample solution to interact with solute n- or π-electrons; the *s* constant to the difference in capacity of the solvated sorbent and sample solution to take part in dipole–dipole and dipole-induced dipole interactions; the *a* constant is a measure of the difference in hydrogen-bond basicity of the solvated sorbent and the sample solution; the *b* constant is a measure of the difference in hydrogen-bond acidity of the solvated sorbent and sample solution; and the *m* constant is a measure of the relative ease of cavity formation for the solute in the solvated sorbent and sample solution together with contributions from dispersion interactions that fail to cancel when the solute is transferred between phases. For any sampling system, the system constants can be obtained by multiple linear regression analysis of experimental retention properties acquired for a group of varied solutes with known descriptors. The solvation parameter model is strictly applicable to neutral compounds and ionizable compounds in their neutral form. Ionization tends to reduce retention compared to the neutral form of the solute in SPE from aqueous solution.

A plot of the system constants as a function of solvent composition provides a system map such as the one shown in Fig. 12.11 [127]. System maps are the most

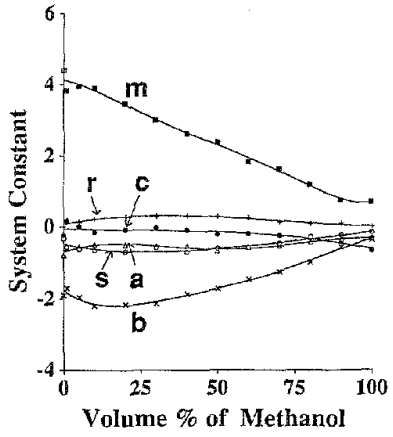


Fig. 12.11. System map for a heavily loaded octadecylsiloxane-bonded silica sorbent with methanol-water as the sample solvent. (From Ref. [127]; copyright Elsevier).

TABLE 12.9

System maps for selecting SPE conditions for aqueous samples using the solvation parameter model

Sorbent*	Type	Solvent	Ref.
IST C-18 (HL)	Octadecylsiloxane-bonded silica (high carbon loading)	Methanol	142
JTB C-18 (LL)	Octadecylsiloxane-bonded silica (light carbon loading)	Methanol Acetonitrile Tetrahydrofuran	132,136
Empore C-18	Octadecylsiloxane-bonded silica particle-loaded membrane	Methanol	151
SPEC-C18AR	Octadecylsiloxane-bonded silica particle-embedded membrane	Methanol	28
JTB C-4	Butylsiloxane-bonded silica	Methanol 2-Propanol Acetonitrile Tetrahydrofuran	150
JTB CN	Cyanopropylsiloxane-bonded silica	Methanol 2-Propanol Acetonitrile Tetrahydrofuran	147,149
JTB DIOL	Silica-based spacer bonded propanediol	Methanol 2-Propanol Acetonitrile Tetrahydrofuran	148
PLRP-S	Poly(styrene-divinylbenzene)	Methanol 2-Propanol Acetonitrile	146
PGC	Porous graphitic carbon	Methanol	77

*IST = International Sorbent Technology; JTB = J.T. Baker; PLRP-S = Polymer Laboratories; PGC = Hypercarb; Empore = 3M; and SPEC = Ansys.

useful approach for method development. The individual system constants change smoothly with solvent composition and can be fit to simple linear or polynomial functions for computer-aided calculation of sampling properties. Once generated the system maps are permanent and can be used to estimate sample-processing conditions based on Eqs. (12.7) and (12.10) for any analyte whose solute descriptors are known or can be reasonably estimated.

In general terms there are three regions of the system map of interest for method development in SPE. The left-hand side of the map, corresponding to low organic solvent, is the region of interest for establishing a safe sampling volume. From system maps for different sorbents the preferred sorbent for the isolation step can be identified. The intermediate region of the system map is of interest for selection of the rinse solvent. The right-hand side of the system map is the region of interest for selecting solvent compositions for the elution of analytes in a small solvent volume. A number of system maps are available for different sorbents and solvent compositions as indicated in Table 12.9.

TABLE 12.10

System constants for the estimation of solute retention in solid-phase extraction for aqueous samples (water or 1% v/v methanol in water)

Sorbent*	System constants					
	m	*r*	*s*	*a*	*b*	*c*
IST C-18 (HL)	4.39	0	0	–0.79	–1.90	–0.27
JTB C-18 (LL)	3.92	0	–0.11	–0.54	–1.53	–0.90
IST C-8	4.91	0	–0.84	–1.43	–2.27	–0.54
IST CHX	3.94	0	–0.32	–0.83	–1.98	–0.31
IST CH	3.22	0.35	0	–0.92	–1.61	–0.37
JTB C-4	3.36	0	0	–0.46	–1.53	–1.38
JTB CN	2.06	0.53	0	–0.51	–1.45	–0.88
JTB DIOL	1.57	0.61	0	–0.45	–0.80	–1.05
PLRP-S	5.22	0.84	–0.49	–1.39	–4.01	–0.18
Oasis HLB	3.48	1.16	0	0	–2.94	–0.32
PGC	5.14	0.82	0.36	0	–2.76	–2.32

*IST = International Sorbent Technology; JTB = J. T. Baker; PLRP-S = Polymer Laboratories (styrene-divinylbenzene porous polymer); Oasis HLB = poly(divinylbenzene-co-N-vinylpyrrolidone); PGC = Hypersil porous graphitic carbon (Hypercarb); HL = high loading; LL = light loading; CHX = cyclohexanesiloxane-bonded; CH = phenylsiloxane-bonded; CN = cyanopropylsiloxane-bonded; and DIOL = spacer bonded propanediol.

Sorbent selection is based on identifying an appropriate sorbent that provides an adequate breakthrough volume for the analytes of interest. The breakthrough volume can be calculated from Eq. (12.7) when the parameters for log QV_M are known or confidently estimated, as is generally the case, and only the retention factor need be calculated using Eq. (12.10) [132]. The system constants for a wide variety of sorbents used for SPE with water or water containing 1% (v/v) methanol as the sample solvent are summarized in Table 12.10. Given that only system constants with a positive sign contribute to sorbent retention we can obtain some general insight into the factors responsible for sorbent extraction from aqueous solutions. For all sorbents the cavity/dispersion term (*m* system constant) is most important for promoting retention. All sorbents are at least as competitive as water for electron lone pair interactions ($r = 0$) and in some cases these interactions contribute favorably to retention (r is positive), if only in a minor way compared to the cavity/dispersion term. Oasis HLB is the exception, having a large positive r system constant, which together with the favorable cavity/dispersion term is the source of its unique selectivity and high retention capacity. In general, polar interactions favor transport in the sample solution and result in smaller breakthrough volumes. The exception is the carbon-based materials, which are the most competitive with water for dipole-type interactions. This is the distinguishing feature of carbon-based sorbents compared to porous polymer and chemically bonded silica sorbents and combined with favorable cavity/dispersion and electron lone pair interaction terms is the

TABLE 12.11

System constants for retention in liquid–solid chromatography with organic mobile phases

Stationary phase*	Mobile phase	System constants				
		m	*r*	*s*	*a*	*b*
DIOL	Hexane	–1.05	0	1.63	2.10	3.86
AMINO		–0.85	0	1.40	1.65	3.81
CYANO		–0.37	0	1.88	2.47	0.99
Silica	Hexane–methanol	–0.83	0	1.06	2.23	1.56
DIOL	(99:1)	–0.85	0	1.07	2.37	1.47
AMINO		–0.72	0	0.94	2.94	1.20
CYANO		–0.61	0	0.95	1.86	1.15
Silica	Hexane–methyl	0	–0.86	1.67	1.84	3.00
CYANO	*t*-butyl ether (95:5)	–1.20	0	1.43	1.10	2.80
Silica	Hexane–methyl	–0.46	–0.21	1.10	1.16	3.02
CYANO	*t*-butyl ether (80:20)	–1.08	0	1.01	0.53	2.26

*AMINO = 3-aminopropylsiloxane-bonded silica; CYANO = 3-cyanopropylsiloxane-bonded silica; and DIOL = spacer bonded propanediol siloxane-bonded silica.

root of their high retention of polar compounds and complementary selectivity to the other sorbents. For the chemically bonded sorbents dipole-type interactions are of limited importance (*s* is either zero or small and negative). The cyanopropylsiloxane-bonded and spacer bonded propanediol sorbents have small values for the cavity/dispersion term compared to other chemically bonded sorbents indicating greater cohesion and a lower retention capacity. The greater cohesion of these sorbents easily eclipses their capacity for selective polar interactions with the result that they are less effective for the isolation of polar analytes from water than the alkylsiloxane-bonded sorbents. None of the sorbents are competitive with water for hydrogen-bond interactions except for carbon-based sorbents. The general difficulty in isolating hydrogen-bond bases arises because the *b* constant for chemically bonded and porous polymer sorbents is quite large and negative. The system properties most important for controlling retention are the high cohesive energy and hydrogen-bond acidity of water for which the solvated sorbents compete to varying extents but fail to dominate. Ironically, these properties are in opposition with respect to sorbent retention. The high cohesive energy of water promotes retention while the hydrogen-bond acidity of water is the principle reason for low retention of hydrogen-bond bases. The equation constant (*c* term) is not related to fundamental properties of the analytes but is important for estimating retention. It is a complex combination of factors, one of which is the phase ratio for the sampling system when the retention factor or breakthrough volume is used as the dependent variable in Eq. (12.10).

There are only a limited number of models that characterize extraction from organic solvents, all of which have been obtained by liquid–solid chromatography (Table 12.11) [148,158–161]. The driving force for retention under normal

phase conditions is the capacity of the solvated sorbent for polar interactions. Increasing solute size generally reduces retention, in contrast to reversed-phase extraction where the m system constant is invariably positive and the most important term controlling solute extraction. Electron lone pair interactions are unimportant for many applications. The main contribution to retention by the 3-aminopropylsiloxane-bonded sorbent is the hydrogen-bond basicity of the solvated sorbent (a constant). For the 3-cyanopropylsiloxane-bonded sorbent it is the capacity of the solvated sorbent for dipole-type interactions (s constant). For the spacer bonded propanediol siloxane-bonded sorbent it is the capacity for hydrogen-bond interactions, particularly as a hydrogen-bond acid (b constant), combined with a significant capacity for dipole-type interactions (s constant) that are important. Silica is a strong hydrogen-bond acid and significantly hydrogen-bond base and dipolar. The range of sorbent selectivity variation is much greater than observed for extraction of aqueous solutions and is very strongly dependent on the composition of the sample solvent. This is seen in the large changes in the system constants observed for hexane and hexane containing 1% (v/v) methanol. For this reason it is unwise to attempt to reduce retention properties to a sorbent property alone. The limited data available indicates that it will be difficult to obtain reasonable breakthrough volumes for analytes that lack polar functional groups and that breakthrough volumes will change significantly with small changes in the composition of the sample solvent. Although the solvation parameter model can model retention on chemically bonded sorbents using organic solvents without apparent difficulty it probably does not correctly account for solute size effects and site-specific interactions for inorganic oxide sorbents [158].

12.5 METHOD DEVELOPMENT

12.5.1 Method and mode selection

Sorbent selection is based on considerations summarized in Fig. 12.12. The sample solvent (aqueous or organic), the analyte type (non-polar, polar or ionized), and if ionized (strong or weak acid or base) provides a logical guide for method selection. Organic compounds soluble in polar organic solvents but difficult to dissolve in solvents of intermediate polarity, if they can be reconstituted in aqueous solution can be extracted in the reversed-phase mode.

The same sorbents used for cartridge SPE are generally used for disc extraction, but since discs are used for a narrower range of applications at present, the number of frequently used sorbent types is fewer, Table 12.12. The majority of disc applications proposed so far are for aqueous samples.

The sample volume is generally selected to conform to the needs of the instrumental detection step, and as these instruments have improved in sensitivity sample volumes have decreased in size. Regulatory authorities often indicate action levels in concentration units, which can also be used to define an

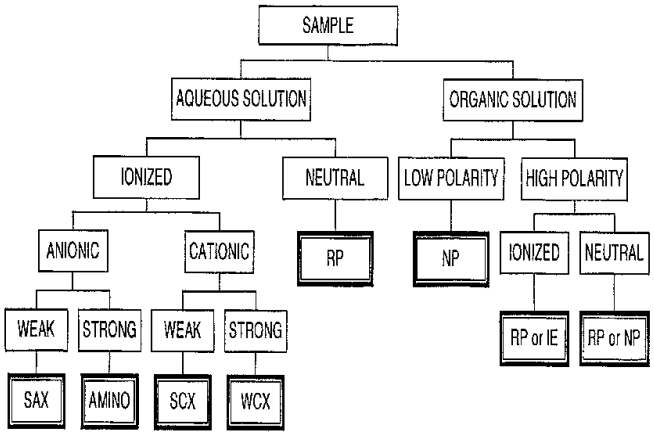


Fig. 12.12. Method selection guide for the isolation of organic compounds from solution. SAX = strong anion exchanger; SCX = strong cation exchanger; WCX = weak cation exchanger; RP = reversed-phase sampling conditions; NP = normal-phase sampling conditions; IE = ion-exchange sampling conditions. (From Ref. [127]; copyright Elsevier).

adequate sample volume for analysis. The sample volume that can be processed by a particular SPE device depends primarily on the breakthrough volume of the analyte, the concentration of the analyte matrix, sample flow rate, and the sorbent mass. SPE cartridges are available in a range of sizes containing from about 35 mg to 10 g of sorbent with the 100 mg and 500 mg sorbent cartridges being the most widely used for extraction and the larger cartridge sizes for sample clean-up. As a rough guide the sample capacity of a SPE cartridge is about 1–5% of the sorbent mass. The parameters of interest for selecting a disc for a particular application are summarized in Table 12.13. The large diameter discs are used for processing large sample volumes in an acceptable time. Small discs are used for processing small sample volumes and to recover analytes in a small solvent volume to eliminate the need for solvent evaporation prior to analysis. Small discs are frequently used in clinical, forensic and pharmaceutical analysis.

12.5.2 Sample processing considerations

Sample processing involves four distinct steps. Initially, the sorbent is conditioned with solvent to remove impurities and to facilitate sorption of the analytes. The conditioning step is critically important for processing aqueous samples using particle-loaded membranes. The high surface tension of water combined with the microporosity of the discs results in slow and uneven flow through the discs and low analyte recovery if the discs are not first conditioned with an organic solvent. The conditioning solvent is then replaced with the same solvent as the sample solvent and the sample passed through the sampling device at a controlled flow rate. For large sample volumes a small amount of organic solvent (1% v/v) is added to aqueous samples to maintain a constant sampling velocity. This usually has little influence on the breakthrough volumes

TABLE 12.12

Sorbent selection for disc applications

Sorbent	General applications
Octadecylsiloxane- and octylsiloxane-bonded silica	Largely used for extracting aqueous solutions. Applications dominated by octadecylsiloxane-bonded sorbents. Many applications to the extraction of non-polar and moderately polar pesticides (all classes), herbicides, phthalate and adipate esters, polycyclic aromatic compounds, food additives, pharmaceutical compounds, hydrocarbons and grease, etc. A large number of approved regulatory methods for water analysis.
Poly(styrene-divinyl-benzene)	Used for compounds poorly extracted by octadecylsiloxane-bonded silica sorbents because of high water solubility (polar pesticides, herbicides, phenols and pharmaceutical compounds). Higher recoveries of neutral polar compounds claimed for polar group functionalized sorbents because of better surface contact with aqueous samples.
Activated carbon	Applications similar to poly(styrene-divinylbenzene) but less frequently used. Methods proposed for triazine herbicides, some polar pesticides and N-nitrosodialkylamines.
Mixed mode	Usually co-bonded alkylsiloxane and benzenesulfonic acid groups (or sorbent mixtures) used predominantly in toxicological and pharmaceutical analysis for the simultaneous isolation of drugs and their metabolites. A number of established methods for drugs of abuse (marijuana and cocaine metabolites, amphetamines, phencyclidine and opiates) in biological fluids.
Sulphonic acid and quaternary amine ion exchangers	Isolation of acidic and basic compounds as well as some metals. Many methods for acidic herbicides and pesticides in water and basic drugs in biological fluids. Hydrogen-ion and metal-loaded cation exchangers available for clean-up of samples analyzed by ion chromatography and capillary electrophoresis.
Crown ethers	Commonly used for the isolation of precious metals (Pd, Pt, Rh) and radionuclides (Cs, Sr, Pb) from high concentrations of other metals to determine environmental burden and for dating geochemistry samples. Other applications include the removal of base metal impurities (Bi, Sb, Fe, Pb, Bi, Cu, Hg) from refinery streams, plating baths, etc.

TABLE 12.13

Guide to the physical properties of particle-loaded extraction discs

Property	Disc diameter (mm)					
	4	7	10	25	47	90
Surface area (cm^2)	0.13	0.38	0.80	4.9	17	64
Bed mass (mg) (silica-based sorbents)	4	10	25	140	500	1850
Flow rate (ml/min)	0.5	1.5	3	20	60	250
Elution volume (ml)	0.15	0.25	0.5	3	10	35
Typical sample volume (ml)	<1	<5	<25	<250	<1000	<5000

except for some porous polymer sorbents, which selectively absorb the organic solvent changing sorbent selectivity and the system phase ratio [72]. Porous polymer sorbents, such as poly(divinylbenzene-co-N-vinylpyrrolidone), that are solvated by water alone, allow samples to be processed without a conditioning step. A sample processing solvent is not usually required for small sample volumes.

Optionally, after the sample is processed, the sorbent can be rinsed with a weak solvent to displace undesired matrix components from the sorbent without displacing the analytes. Finally the analytes of interest are eluted from the sorbent in a small volume of strong solvent for subsequent determination. The drying step between processing aqueous samples and eluting the retained analytes with a water-miscible organic solvent is also important. Water retained by the sorbent (and the support structure when discs are used) contaminates the elution solvent and may cause difficulty if the solvent is to be reduced in volume or the analytes analyzed by gas chromatography. Water may also reduce the efficiency of the elution step, particularly for solvents only partially miscible with water. Drying, by suction or vacuum desiccation, is usually sufficient to remove water trapped in the pores, but excessive drying can result in low analyte recovery from vaporization or inefficient elution. General advice for establishing optimum sample processing conditions for cartridge and disc sampling devices is summarized in Table 12.14. A generic outline for processing samples using disc technology is outlined in Table 12.15. This can be re-scaled for different disc and sample sizes using the data in Table 12.13.

12.5.3 Computer-aided methods

In spite of its relative maturity computer-aided strategies for SPE method development are still uncommon. A significant contributing factor to this situation has been the poor understanding of the theoretical principles of SPE until recently and the low status accorded to sample preparation compared with separations by many professional scientists best able to develop such theories. Expert systems have been described for the optimization of sample processing conditions for selected drugs in biological fluids [162,163], but there is little evidence from the literature that expert systems are widely used at present. It was shown recently that all sample-processing steps in SPE are amenable to computer simulation [132]. Seibert and Poole used Eq. (12.7) and the solvation parameter model, Eq. (12.10), to predict the sample processing conditions for the isolation of estrogens from urine. System maps were used to determine breakthrough volumes for sample processing, the composition and volume of rinse solvents for matrix simplification, and the composition and volume of elution solvents. A sample volume of 45 ml was required to provide sufficient estrogens for convenient analysis by gas chromatography with flame-ionization detection. Estriol, the least retained of the estrogens, had a predicted breakthrough volume greater than 45 ml on an octadecylsiloxane-bonded silica sorbent cartridge with

TABLE 12.14

Sample processing parameters and their influence on recovery by solid-phase extraction

Conditioning solvent (typically 3–5 bed volumes)
- Ensures reproducible retention and flow. Critical step for particle-loaded membranes
- Helps to minimize contamination of extracts by sorbent impurities
- Replace by sample solvent before processing sample

Flow rates (typical range 0.2–1.5 mm/s)
- More critical for cartridges than discs due to their variable and heterogeneous packing density (channeling)
- More critical when the sample volume exceeds the breakthrough volume as typical sampling devices provide too few theoretical plates for flow independent retention

Sample properties
- Dilute viscous samples with a weak low viscosity solvent to reduce sample processing time
- Remove excessive particle matter by filtration or centrifugation to maintain a constant sample-processing rate.
- Add small volume of organic solvent (1–3% v/v) to large volume water samples to ensure sorbent remains solvated and to maintain a constant (fast) sample-processing rate. Important for particle-loaded membranes
- Adjust pH to reduce ionization of weak acids and bases for reversed-phase sampling
- Maintain approximately constant ionic strength for samples and standards when using reversed-phase sampling conditions. Ionic strength is a critical parameter for ion-exchange extraction
- Deproteination of biofluids may be required for acceptable recovery of low molecular mass analytes for reversed-phase sampling
- Precipitation of inorganic acids (sulphate, phosphate, etc.) by barium hydroxide is sometimes required for acceptable recovery of organic acids from biofluids using ion-exchange extraction

Drying time (typically 1–5 min, but sometimes considerably longer)
- Sufficient to remove all sample solvent trapped in the sorbent pores
- Excessive drying may result in low recovery of analytes from evaporation or retention in poorly solvated regions of the sorbent

Rinse solvent (optional)
- Small volume of intermediate strength solvent to elute matrix components. Analytes remain immobilized on the sorbent
- Biological fluids, plant extracts and soil extracts often require a rinse step but surface waters may not

Eluting solvent (ideally 2–3 bed volumes but often larger)
- Should be a strong solvent able to displace all analyte from the sorbent in a small volume
- Normally should be volatile and miscible with the sample solvent

a sample solvent containing less than 25% (v/v) methanol. A methanol-water mixture was selected to minimize matrix sorption during sample application. A volume of 6 ml of 40% (v/v) aqueous methanol was identified as an appropriate rinse solvent for matrix simplification. The solvent composition required to quantitatively elute the estrogens, predicted for estrone as the most retained estrogen, were three cartridge hold-up volumes of methanol. The good agreement between model predictions and experiment (recovery 95–101% with an RSD = 3.5%, n = 5) was taken as a validation of the approach. From the system

TABLE 12.15

Generic guide for sample processing using solid-phase extraction discs. In this example a 47-mm disc and a 1-l water sample are used for illustration

Decontamination	With disc installed in filtration apparatus (or cartridge) rinse with 10 ml of solvent (acetonitrile) by allowing the solvent to soak into the membrane for a few minutes and then remove by suction.
Condition	As described above using 10 ml of methanol. Before the last drop of methanol has been sucked through the disc add 10 ml of deionized water. The disc should not be allowed to suck dry until after the sample has been processed.
Sample	The sample containing 1% (v/v) methanol is passed through the disc with a suitable reservoir in place. Filter aid or a prefilter may be required for samples with a heavy burden of particulate matter. The sample is processed at a flow rate between about 50 and 100 ml/min using a vacuum of about 10–20 mm Hg.
Drying	Bulk water is removed from the disc and sampling apparatus by sucking air through the disc under full vacuum for about 3 minutes. Volatile compounds may be lost.
Recovery	The analytes are recovered by passing two 5 ml volumes of acetonitrile through the disc. The first volume of acetonitrile is allowed to soak into the disc for a few minutes and then gently sucked through the disc. Without letting the disc run dry the second volume of solvent is added to the disc and sucked through the disc. The disc is then allowed to suck dry.

maps given in Table 12.8 thousands of methods could be simulated without resort to initial trial-and error experiments for compounds with known or estimated solute descriptors.

12.6 AUTOMATION AND COUPLED-COLUMN SYSTEMS

SPE lends itself to automation to an extent that has been difficult to achieve using LLE procedures [1,4,166]. A major impetus for the development of automated laboratory procedures has come from increasing demand for high throughput methods in clinical, pharmaceutical and environmental laboratories. Automation is viewed as an essential feature of achieving this goal by reducing time and cost of labor while improving method accuracy and precision, reducing assay tedium and enhancing safety by limiting exposure to chemicals and pathogens. In the most favorable case automated procedures provide for out-of-hours operation and require a minimum of operator intervention. This is not always achieved, however.

Automated methods can be considered in three categories. The most common are semi-automated instruments where operator intervention is required at some stages during the extraction. These instruments are designed to reduce the tedium of repetitive manual operations. They may increase sample throughput and improve accuracy and precision, but are really method assistants.

Workstations, on the other hand, are dedicated instruments that usually carry out all stages of the SPE method without intervention, and are usually easily coupled to separation instruments. Most early versions, however, operated in a serial sampling mode or semi-batch mode and offered limited improvement in sample throughput. The best of these systems can extract between 25–50 samples per hour, but the majority are nearly an order of magnitude slower than this. For coupled-column systems this was not a problem when the separation was slow, but with increasing use of fast separation methods and mass spectrometric detection, which is less demanding of extraction selectivity, this was no longer adequate. Really high sample throughput was achieved by parallel sample processing using workstations designed around the 96-well plate format. The best of these systems can extract up to about 400 samples per hour, but are usually somewhat limited in the sample volume that can be handled, and are mainly used in clinical and pharmaceutical laboratories, where large sample volumes are rarely required for quantification of target analytes. A higher level of automation can be achieved using robotic systems that are capable of many activities besides SPE (e.g. weighing, centrifugation, filtration, capping, evaporation, bar code reading, etc). These can often process the sample from an earlier stage in the analysis through to injection of extracts into the separation system.

Automation is probably not a high priority for all laboratories. In the absence of large sample numbers requiring a short turn around time fully automated systems are difficult to justify. The best of the modern systems use convenient graphical interfaces that are easy to use but earlier and some existing systems require specialized knowledge of computer programming and electronics for operation and maintenance. Carryover from the sharing of common modules by the samples can limit performance and reduce accuracy through contamination and limit throughput by the need for repetitive programmed rinse procedures. Systematic errors arise when the instrument fails to take account of sample properties, such as incomplete withdrawal of sample volumes due to changes in viscosity, precipitation or clotting. Sample stability can be a problem for some samples and conditions, for example, when samples are delayed for various times prior to being processed. Differences in liquid management, for example, the use of positive pressure versus vacuum, often means that manually developed procedures do not function well on automated systems without extensive re-optimization. On the other hand, automated workstations provide a more controlled environment for method development with the possibility of running larger sets of trial experiments in a shorter time to identify rugged extraction conditions.

12.6.1 Coupled-column techniques

On-line coupling of SPE-LC is quite straightforward and is in common use [4,6,21,86,94,98,165–167]; SPE-GC is somewhat more complicated but has been achieved in a robust fashion in the last few years [20–22,168]; and SPE-CE is

feasible but largely restricted to use in research laboratories for the present [169–171]. The majority of applications are for aqueous samples reflecting the main use of the technique in clinical, pharmaceutical and environmental laboratories for the analysis of biological matrices and surface waters. Typical sample volumes for SPE-LC are 1–10 ml while larger volumes, 10–1000 ml, are required for environmental applications. For SPE-GC sample sizes are often an order of magnitude smaller because of the greater sensitivity provided by typical gas chromatographic detectors. The design of precolumn sampling cartridges is similar for SPE-LC and SPE-GC as are the sorbents used. Low-specificity sorbents are used for general applications, mainly octadecylsiloxane-bonded silica gel and porous polymers [167,169,172], and class-specific sorbents, particularly restricted access materials and immunosorbents, for selective extraction of target analytes [96–101,173–177]. Restricted access materials have gained acceptance for the direct analysis of drugs in biological fluids and the analysis of polar pesticides in the presence of humic acid in surface waters. Breakthrough volume considerations are generally unimportant for sampling biological fluids, since only small sample volumes are required for adequate detection of target analytes but for environmental applications this is a critical parameter and dominates sorbent selection. High surface area porous polymers and to a lesser extent graphitized carbon sorbents, are generally used for environmental applications [6,43,178–181], but no single sorbent is suitable for all applications.

The choice of precolumn dimensions and sorbent properties is a compromise between the need for sufficient retention to accommodate the desired sample volume and efficient analyte desorption for transfer to the separation column without unacceptable loss in its separation power. Typical precolumns are 10–30 mm long with internal diameters of 1–3 mm when used with standard 4–4.6 mm internal diameter separation columns in liquid chromatography or 2–20 mm long and 1–4.6 mm internal diameter for coupling to gas chromatography. These columns contain about 10–50 mg of sorbent with an average particle diameter in the range of 10–40 μm. Less frequently precolumns are prepared from several small particle-loaded membrane discs assembled to form a continuous bed. For automated operation the precolumn must be either reusable or automatically exchangeable.

The on-line coupling of precolumn and separation column in liquid chromatography is achieved using a single 6-port automated switching valve with flow paths indicated in Fig. 12.13, or by using two automated valves for additional versatility in automating a wider range of sample processing steps. The control system has to provide for regeneration and conditioning of the precolumn, loading of the sample, rinsing the precolumn to minimize matrix constituents and transfer of the analytes to the analytical column for separation. Usually the sample processing steps occur concurrently with the separation of the previous sample. All events are programmable and optimized by time and flow control. For strongly retained analytes, backflushing the precolumn is often the best approach for solvent desorption. The solvent used for desorption is usually the

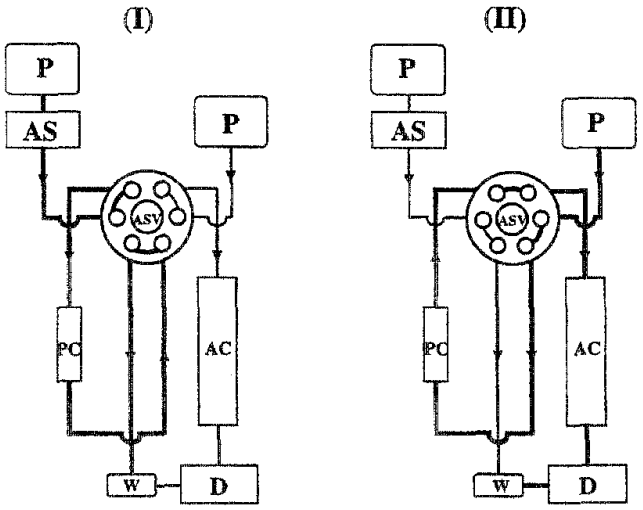


Fig. 12.13. Arrangement for automated coupled-column switching in liquid chromatography. Position (I) is for sample application and fractionation and position (II) for transfer of extracted analytes and subsequent separation. P = pump, AS = autosampler, ASV = automated switching valve, PC = precolumn, AC = analytical column, D = detector and W = waste. (From Ref. [94]; copyright Elsevier).

same or a weaker solvent than the mobile phase used for the start of the separation. The analytes are also distributed over a significant length of the precolumn and their transfer usually occurs in a solvent volume that is too large and perhaps too strong to avoid additional band broadening compared with conventional injection techniques. The precolumn dimensions (short column of smaller internal diameter than the separation column), sorbent particle size (small particle diameter) and the retention capacity of the sorbent for the analytes (weak retention capacity) are optimized to assist in minimizing band broadening due to the transfer process. On the other hand for successful sampling the sorbent should have strong retention properties, the precolumn should be as large as practical and packed with coarse particles to assist in sample application when large volumes are used. For environmental samples, sample volumes usually exceed 100 ml and sample application rates of 5–15 ml/min are common. The flow resistance of the precolumn is an important consideration for these sampling conditions. The criteria for extraction and desorption are generally opposite and practical operating conditions are always a compromise.

In most cases preconcentration of analytes occurs with simultaneous matrix accumulation. Matrix simplification can be achieved through the sampling process, the use of a rinse solvent prior to analyte transfer, and selective transfer of front-, heart- or end-cuts of the desorbed sample extract. Dual precolumn systems allow solvent exchange and separate trapping of analytes with a wide range of breakthrough volumes. The first column of a dual precolumn system can be used to retain strongly sorbed matrix components that would contaminate the second precolumn or interfere in the separation of the transferred extract.

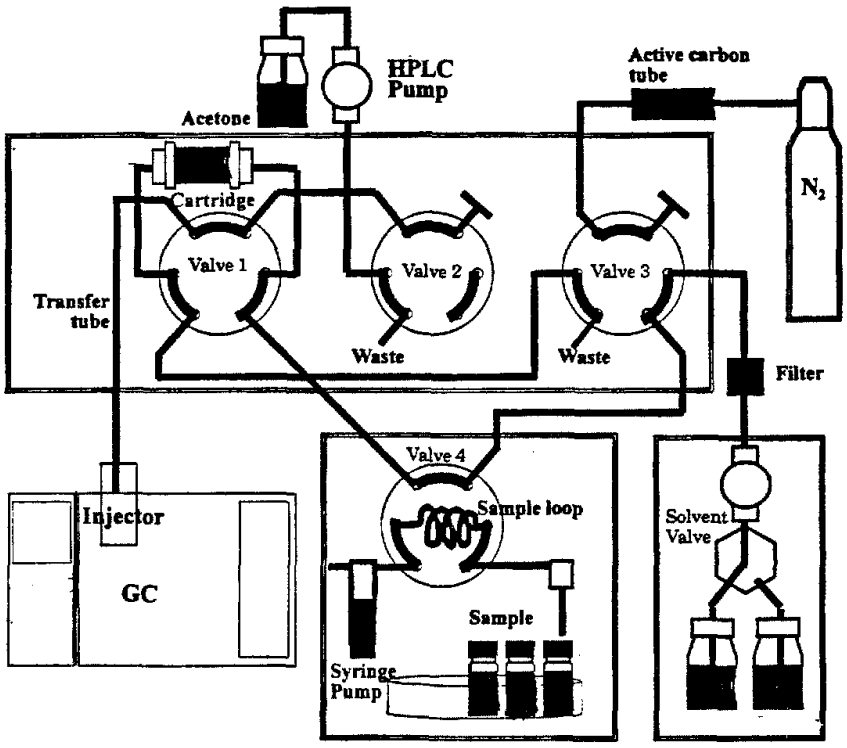


Fig. 12.14. Typical valve switching arrangement for automated SPE-GC. (From Ref. [168]; copyright Elsevier).

Automated SPE-GC coupling is achieved in a similar manner to SPE-LC. The precolumn sample processing and clean-up steps are the same except that sample volumes are often smaller and solvent selection for desorption is limited to solvents compatible with GC injection techniques, which are generally different to the selection criteria for LC [20-23]. A suitable apparatus for automated SPE-GC is shown in Fig. 12.14 [168,182–184]. The desorption solvent is transferred to the gas chromatograph via a narrow bore deactivated fused silica capillary to a standard GC injector connected to a retention gap and possibly retaining precolumn and an early solvent vapor exit.

For on-column injection transfer occurs under conditions of partial concurrent solvent evaporation with the solvent vapor exit initially open during transfer and closed just before completion of the evaporation process. An efficient precolumn drying process is important, since even a few percent of water absorbed by the desorption solvent will cause destruction of the deactivated retention gap. Apart from purging with nitrogen at room temperature, which is a slow process, a drying cartridge containing anhydrous sodium sulfate or silica gel can be inserted between the SPE cartridge and the inlet of the gas chromatograph [184]. The loss of partially trapped analytes is more critical in on-line SPE-GC than for conventional injection techniques because of the varying analyte distribution in the transfer solvent. The introduction of a small volume of organic solvent, presolvent, immediately in front of the extract to ensure that

a solvent film is already present in the retention gap when extract transfer starts, can be used to minimize this loss.

Loop-type interfaces with partial concurrent solvent evaporation in a long retention gap are more robust and easier to automate than the on-column interface but are restricted to the transfer of analytes of low volatility [20, 185–187]. The interface is equipped with a 6-port and a 14-port switching valve. The 6-port valve is used to divert the carrier gas either to the gas chromatograph or to the 14-port valve, on which the SPE cartridge and two loops for storing organic solvent are mounted. The first loop contains the presolvent and is usually of smaller volume (e.g. 50 μl). The second loop contains the solvent used to desorb the analytes from the SPE cartridge and to transfer them to the gas chromatograph. After the sample processing steps are completed and the cartridge dried, both valves are switched simultaneously and the solvent vapor exit opened. The carrier gas pushes the contents of both loops, the second loop via the SPE cartridge, into the retention gap. Both valves are switched to the load position when a preselected pressure drop corresponding to near completion of the evaporation process is recorded and the solvent vapor exit closed after a selected delay time. The transfer is carried out at a temperature just above the solvent boiling point.

12.7 CONCLUSIONS

Solid-phase extraction is now a well-established sample preparation technique with an ever-widening applications base. The methodology continues to evolve through changes in format more than principle. The driving forces being the desire to simplify the sampling process or enhanced automation possibilities. Advances are expected in sorbent chemistry, particularly class-specific sorbents for the isolation and clean-up of target analytes in complex matrices. Advances are also expected in the use of computer-aided method development strategies as the logical replacement for tedious trial-and-error procedures commonly used today. Further development of automated SPE systems can be expected in those laboratories where high sample throughput or round-the-clock process monitoring is important. In addition, SPE technology has defined new opportunities for use in passive sampling, storage and preservation of extracts, field sampling, and as a biometric indicator. Fundamental research into the development of SPE techniques and their potential applications is likely to continue at an increased pace in the next decade as analysts attempt to capitalize on the above opportunities.

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