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on the occasion of his 80th birthday (tribute)

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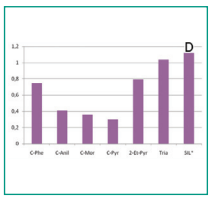

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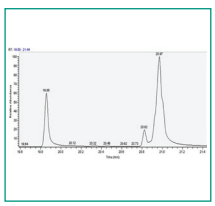

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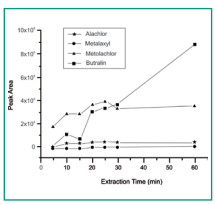

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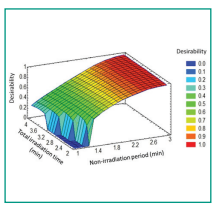

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EDITORIAL

Happy Birthday

Professor Harold M. McNair
80 years old

This special issue, as well as the previous issue of *Scientia Chromatographica* yields a tribute to Professor Harold McNair, Emeritus Professor at the Virginia Polytechnic Institute and State University, Blacksburg, VA, USA, on the occasion of his 80th birthday. In addition to being an internationally recognized scientist in the separation science arena, Professor McNair is a member of the Editorial Advisory Board of *Scientia Chromatographica* since its conception and first release.

The editors, members of the Editorial Board and readers of *Scientia Chromatographica* are happy to greet him on this occasion and wish him many happy years to come!

Fernando M. Lanças
Editor

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TRIBUTE

Harold M. McNair. The Seventh Horizon.

"You should think about working in chromatography in the future, Michael. There are great possibilities in this field for chemists."

Professor Keith Bowden, University of Essex, 1968.

Michael P. Henry

40 Stuartholme Road, Bardon,
Queensland, Australia, 4065.

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Abstract

This paper is a brief account of my 35-year friendship with Harold and Marijke McNair. It was a chance meeting with Harold that resulted in the total refocusing of my career in chemistry. Concurrent with that, several decisions were made that changed completely the lives of my wife and two daughters. I write of my experiences in Harold's lab at Virginia Tech and subsequently his support of several successful job applications in the USA that transformed my life as a chemist and in many other ways. My varied career in chemistry can be characterized in one way by a series of broad horizons that fortuitously opened up to me and maintained my fascination with this science over many decades. My first meeting with Harold was the seventh horizon.

1. Introduction

A chance meeting with Harold McNair altered completely the direction of my family's life. Prior to that meeting my wife Victoria and daughters Katya and Justine had no plans to move out of the sub-tropical city of Brisbane, Australia. It was assumed that I would work as a lecturer in chemistry at the Queensland Institute of Technology till retirement, Vicki would become a lawyer and my daughters would obtain worthy careers, perhaps marry Australians and settle down in this country. But that was not to be.

Several of my chemical colleagues introduced me to Harold McNair during his visit to Brisbane in 1977, purely on the basis that he and I had certain principles of audio-visual teaching ideas in common. At the time my forward-looking department was interested in introducing HPLC to its undergraduate students and Harold offered a visiting professorship at Virginia Tech to train a faculty member in this technique. I applied to my department for the post and was successful.

On the basis of my HPLC experience in Harold's lab and elsewhere in the USA, plus the deep friendship that developed between my family and his, I determined to obtain employment in America in this field. I therefore applied to J. T. Baker Chemical in New Jersey for a senior scientist position during Pittcon 1980. I heard nothing from Baker Chemical for almost a year.

2. Experimental

2.1. Sabbatical 1978-1979

Prior to applying for the visiting professorship, I discussed this dislocation with my family. Victoria had, as always, fully-formed ideas on such a move, and my six and four year old daughters had little say. So we all moved to Blacksburg, Virginia, where our life changed. Here there was snow, four seasons, the Blue Ridge Parkway, Bluegrass music, a college town, the Carol Lee donut shop, Kroger's supermarket, homecoming queens, marching bands, Gilbert Linkus Elementary School and a large number of wonderful public holidays.

The experience was made immensely richer through our friendship with Harold and Marijke McNair and their three children. We were immediately welcomed by them all and through them we made many other friends and immersed ourselves in the culture of America. Harold frequently invited us to lunches and dinners and other social outings with his students and/or his family. His whole family burned with enormous creative energy. We were content to move into their centre and be carried along by them to places we had never before experienced. Within months Katya and Justine had developed southern accents. In spring 1979 we toured the southern states of the Carolinas, Georgia and Florida, marveling at the food, the languages and the history.

I always felt that I had jumped into the deep end of life in America, but I was buoyed up by the immense energy that pulsed throughout the country. My lazy brain suddenly came to life and the faith that Harold had in me did wonders for my self-confidence.

In mid 1979 I completed my visit in Harold's lab and we all returned to Brisbane, where life went back to where it left off in June 1978. More or less.

This sabbatical year would have been the end of my American adventure, except for two things:

- Harold and Marijke's extraordinary ability as warm and generous hosts, and
- My immediate fascination with the whole field of liquid chromatography.

2.2. Life in Industry?

I had almost forgotten about the job application I had made in 1980.

One sweltering summer morning in Brisbane I received a phone call from Laura Crane, principal scientist in charge of lab product development at Baker. She had spoken to Harold McNair about my suitability. Was I still interested in the job? I said yes and flew to Atlantic City, New Jersey for a 2 days interview to be

conducted after Pittcon 1981. I was hired in April 1981. It was an impossible result achieved by the combined faiths of Laura Crane, Harold McNair and Hal Kaufman, J.T.Baker Vice President of Research.

Before I decided to take up Baker Chemical's offer, there were many family discussions about this move. Now the future of my family – Vicki, Katya and Justine – was about to undergo a major change. Not a simple temporary visit to Virginia, but an indefinite period of migration to the United States, where opportunities were abundant.

I would be leaving a permanent academic job in a familiar country for a temporary position in the chemical industry in a new country. Vicki had a year or so to go of her business degree. Katya was 10 and Justine was 8 years old. All would have to leave their friends and a society that was a cradle of their upbringing. It was an enormous decision to make. In retrospect I do not know how I made the decision to leave Australia. My Department Head at the time, Dr. Stan Dyke, said I was crazy. A recession had gripped the USA in the early 1980's with unemployment at 10%. I was hired on a 12 month temporary work visa, with no guarantee of renewal. Laura Crane had begun the process of acquiring a green card, but my job had to be publically advertised and offered to a suitable American candidate. It was the combined effects of the support from my wife, the friendship of Harold and Marijke McNair and the strong encouragement from Laura Crane and Hal Kaufmann that confirmed my decision.

3. Results and Discussion

3.1. Virginia Tech. 1978-1979.

Back in Blacksburg, Harold arranged for me to teach freshman chemistry classes of several hundred students of many backgrounds and majors. They all seemed very smart, very articulate and very good looking.

Then after an appropriate amount of instruction in the basics of GC and LC using a series of brilliantly designed 35mm slide programs, Harold exposed me to many of his live short courses in these fields of separation science. Eventually I was able to speak on selected basic chromatography subjects to attendees at innumerable short courses throughout the United States. Sometimes I could answer their questions. For the first time in my long career in chemistry instrumentation became a major focus of my work. Unlike an infrared or nuclear magnetic resonance spectrometer, which are adjuncts to synthetic work for example, the instrumentation in HPLC is the entire field of this separation science. I became immediately intrigued by the components of the high pressure chromatograph.

A firm understanding of HPLC was made somewhat easier in Harold's lab when my first experience of the technique was obtained via an extraordinarily simple student system. Pressure came out of a gas bottle, the mobile phase was contained in a helical tube of steel, the injector was a loop device, the column was packed with 10 micron irregular particles of bonded silica, the detector seemed to be composed of a UV lamp a crude flow cell and some ill fitting tubing. The chromatograph was displayed on the simplest of paper rolls by ink-loaded pens, which seemed to dry up at crucial times. There was no computer control over the method, sample access and delivery, instrument set up or data recording. Just a few hand-turned valves and injectors. Eventually your liquid chromatograph became your friend – you got to know its foibles and how to treat it well day after day, producing eye-popping results of outstanding sensitivity and accuracy – eventually. But the one thing that remained something of an unknown was the nature of the stuff inside the HPLC column. After all, this was where the separation occurred. How did they create bonded phases and how did they manage to pack 10-micron and all the way down to 1-micron particles so tightly and evenly into that tube of stainless steel. I wasn't going to find out the intricacies of those processes for some time. And how that came about is a truly remarkable story.

3.2. The Chemical Industry. 1981-2005.

In early September 1981 I left Vicki, Katya and Justine at Sydney airport. I would not see them again for 12 months. I had no idea then of how traumatic that separation was going to be. But I knew I was going to have to justify the faith that Laura Crane had placed in me and I was not sure how I was going to do that. I did know that I was going to need to summon all my knowledge in chemistry and the new horizon of science that had opened up to me during that brief idyll in Blacksburg with Harold McNair three years beforehand. The intensity of my work at J. T. Baker helped overcome the real feelings of grief that I experienced without Vicki, Katya and Justine being there. So I began a 24-year chemical odyssey in America working in New Jersey for 11 years and in three jobs in California over a period of 13 years.

My work at J. T. Baker Chemical reporting to Laura Crane for nine years and finally to John Covington for two years, involved participating in the development of a full range of bonded phases for solid phase extraction and liquid chromatography. In 1992 I left Baker to work with Toxi-Lab under Steve Schultheis (now with Agilent Technologies) in Southern California. This company was known as a developer and supplier of systems for rapid drug analysis using high performance thin layer chromatography. The substrate was fiberglass into which silica and bonded silicas could be enmeshed. The technology the Company had developed for producing large batches of thin layer material was truly remarkable.

After two years with Toxi-Lab I moved north to Applied BioSystems in Foster City, reporting to John Wiktorowicz. There I had the great good fortune to meet Joe Pesek and Maria Matyska, who researched and invented new material for HPLC and capillary electrophoresis. My final position – after two years with Applied BioSystems – was a much longer association with Beckman Coulter under Chan Oh. I worked with the immensely talented Chitra Ratnayake, first on HPLC, then capillary electrophoresis and capillary electrochromatography. While Chitra went on to become Senior Staff Scientist I stayed with the Advance Technology Group. Here I reported to Stephen Pentoney and worked in the field of flow cytometry, devising systems for the rapid analysis of cytokines in biological systems.

In 2005 I returned to Australia with Vicki to live again in the same modest house I bought in 1972 and left in 1981.

3.3. A Family in America.

Vicki, Katya and Justine joined me in late September 1982. We lived on College Hill, Easton, Pennsylvania for ten years. Vicki took on a broad variety of positions in teaching, manufacturing and non-profit organizations; and carried all that experience wherever she and I moved. My daughters were signed on to attend elementary, middle and high schools in Easton, where the going was tough. They survived however and scattered over the globe as they graduated from school and then Universities and took up positions in marketing and teaching.

Katya now lives in Brisbane with her partner and son and daughter. Justine lives in Yonkers, New York with her husband and son and daughter.

4. Conclusions

I suspect that every aspect of my life and that of my family would have been substantially different if I had not met Harold McNair on that fateful day. I owe Harold my immense gratitude for his central role in so comprehensively expanding my career horizons in chemistry. This brief account describes just a few of those horizons. They not only cover a dozen fields of this science but also gave me the opportunity of seeing life in many countries all over the globe.

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Synthesis of stationary phases containing pyridine, phenol, aniline and morpholine via click chemistry and their characterization and evaluation in supercritical fluid chromatography

Melissa Dunkle[1,2]
Caroline West[3]
Alberto Pereira[2]
Steven Van der Plas[4]
Annemieke Madder[4]
William Farrell[5]
Eric Lesellier[3]
Frédéric Lynen[1]
Pat Sandra[1,2*]

[1]Pfizer Analytical Research Centre (PARC), UGent, Krijgslaan 281 S4-bis, 9000 Gent, Belgium
[2]Research Institute for Chromatography (RIC), President Kennedypark 26, 8500, Kortrijk, Belgium
[3]Institut de Chimie Organique et Analytique (ICOA), Université d'Orléans, CNRS UMR 6005, BP 6759, rue de Chartres, 45067 Orléans Cedex 2, France
[4]Department of Organic Chemistry, UGent, Krijgslaan 281 S4-bis, B-9000 Gent, Belgium
[5]Pfizer Global Research and Development, La Jolla, 10770 Science Center Drive, San Diego, CA 92121, USA

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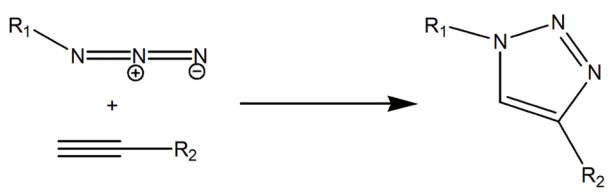
Abstract

Stationary phases containing pyridine, phenol, aniline and morpholine groups were synthesized using copper (I)-catalyzed azide-alkyne cycloaddition *click* reactions. The backbone of the stationary phases was aminopropyl silica. The stationary phases were evaluated in packed-column supercritical fluid chromatography (pSFC) with acidic and basic solutes without addition of additives. The analysis of metoclopromide and its impurities by SFC-time-of-flight mass spectrometry (SFC-TOFMS) on the phenol phase is presented. In the *click* reaction, the 1,2,3-triazole ring is formed and to assess its influence on the polarity/selectivity, the *click* phases were compared to a commercial available 1,2,4-triazole hydrophilic interaction liquid chromatography (HILIC) phase. The phases were also compared to two extensively used stationary phases in SFC namely 2-ethyl pyridine and bare silica. To allow comparison with other phases used in SFC, linear solvation energy relationships (LSER) of the *click* phases were determined.

Keywords: Packed-column supercritical fluid chromatography; Stationary phases; Click chemistry; Linear solvation energy relationships; Pharmaceuticals.

1. Introduction

In recent years, *click* chemistry has become an evermore popular reaction to join small units together in synthesis. A reaction to be termed as '*click* chemistry' must meet the following criteria: the reaction must be modular, wide in scope, give very high yields, generate only inoffensive byproducts that can easily be removed, and be stereospecific. Moreover, the reaction process must include simple reaction conditions, use readily available starting materials and reagents, use of no solvent or a solvent that is benign or easily removed, and have simple product isolation[1,2]. There are several classes of reactions that meet these criteria: cycloadditions of unsaturated species, nucleophilic substitution chemistry, 'non-aldol' carbonyl chemistry, and additions to carbon – carbon multiple bonds[2-4]. For the purpose of this project, focus was emphasized on the copper (I) catalyzed cycloaddition of azides to alkynes, which provides a powerful *click* chemistry tool with remarkably broad scope and exquisite selectivity for the conjugation between an appropriately functionalized terminal alkyne and organic azide, to form a 1,4-disubstituted 1,2,3-triazole ring[5-8]. This reaction has even been termed the best *click* reaction to date[9]. One of the most commonly used methods to generate the active catalyst is to use a copper (II) salt, which is reduced *in situ* with sodium ascorbate to form a copper (I) salt[2-4] (Scheme 1).



Scheme 1. Formation of the 1,4-disubstituted 1,2,3-triazole ring.

This *click* reaction has also made its way into the chromatographic arena. Several publications have reported the use of *click* chemistry for the synthesis of stationary phases for HPLC including reversed-phase, hydrophilic interaction and weak cation-exchange supports[5,6,10-13]. We recently described the synthesis by *click* chemistry of estradiol and testosterone phases for affinity LC of tripodal receptors[14].

Packed-column supercritical fluid chromatography (pSFC), pioneered by Klesper et al.[15], has been around for over 25 years and is intensively used for some specific applications, for example, chiral separations in the pharmaceutical industry. In recent years, its popularity has increased mainly because of its green character. SFC is environmentally friendly and minimizes the use of toxic organic solvents and additives, with their concomitant risks of laboratory worker exposure and disposal problems. The main instrument manufacturers recently introduced new high-performance SFC instrumentation and the technique is presently experiencing a boom as complementary method to reversed-phase LC and HILIC.

Although nearly all HPLC columns have been used in analytical pSFC, silica-based normal-phase stationary phases i.e. bare silica, diol- and cyanopropyl-bonded silica have been mostly applied until ten years ago. In attempts to avoid or reduce the use of acidic and basic additives, dedicated pSFC phases were synthesized and became commercially available. In 2001, Princeton Chromatography (Cranbury, NJ, USA) introduced a 2-ethyl pyridine stationary phase and many basic pharmaceuticals could be eluted with excellent peak shape without addition of an additive. A number of other phases (amide, urea, etc.) were synthesized to provide alternative selectivities while other small companies entered the field by synthesizing SFC dedicated stationary phases e.g. Zymor (Wayne, NJ, USA) and ES Industries (Berlin, NJ, USA). More recently, Waters (Milford, MA, USA) announced a line of Viridis SFC columns (including 2-ethyl pyride silica) and Phenomenex (Torrance, CA, USA) introduced a series of phases specially designed for SFC. A recent review of column developments for SFC can be found in ref [16].

As it has only been in the last few years that *click* chemistry has been used as a means to develop stationary

phases, it is not surprising that stationary phases for pSFC have not yet been developed by this method. The objective of this work was to use the fast, simple and versatile copper (I) catalyzed azide-alkyne cycloaddition reaction to synthesize some new, unique and tailored stationary phases for pSFC. The functionalities selected are pyridine, phenol, aniline and morpholine. Pyridine and morpholine stationary phases are commercially available but the way of linking to silica is completely different. On the other hand, other reasons to synthesize the phases are (i) batch-to-batch variability of commercially available phases making implementation in a drug discovery laboratory problematic, (ii) substantial bleeding of commercial phases in SFC-MS hypenation and (iii) the need for larger quantities of the stationary phases for semi-prep SFC. The phases were evaluated through a study of their interaction capabilities with the help of linear solvation energy relationships (LSERs)[17-19]. The *click* phases have also been compared to commercially available silica and 1,2,4-triazole-silica, both developed for HILIC, and to 2-ethyl pyridine silica.

2. Experimental

2.1. Materials

Propargyl bromide solution (~80% in toluene), morpholine (> 99.0%), 2-ethynyl pyridine (> 99.0%), dimethylformamide (DMF), dichloromethane (DCM), diphenyleneiodonium chloride (DPI), hydrochloric acid, methanol, pyridine, acetic acid anhydride, sodium azide, 6-bromohexanoic acid, and sodium ascorbate were obtained from Sigma-Aldrich, Munich, Germany). 3-ethynylphenol (>95%) and 3-ethynylaniline (98%) were purchased from Acros, Geel, Belgium. Fuji Chromatorex aminopropyl silica (5 µm d_p) was from Fuji Silysia Chemical (Kasugai, Japan), and benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) was obtained from Merck Biosciences (Darmstadt, Germany).

One hundred and twenty seven compounds (Table 1) used for LSER characterization were obtained from a variety of manufacturers. Individual solutions of these compounds were prepared in methanol and stored at 4°C. For details on solute selection, please refer to[19]. The acidic (ibuprofen, fenoprofen, flurbiprofen and ketoprofen), basic (caffeine, theophylline and theobromine) and neutral (cortisone, prednisone, hydrocortisone and prednisolone) model solutes were from Sigma-Aldrich, (Munich, Germany) Stock solutions at concentrations between 1 and 5 mg/L were prepared in methanol and stored at 4°C and then further diluted in methanol. Metoclopromide and its impurities were obtained from LGC Standards (Molsheim, France).

A 2-ethyl pyridine column (25 cm x 4.6 mm i.d., 5 µm d_p) was purchased from Bischoff (Leonberg, Germany), a Cosmosil HILIC triazole column (15 cm x 4.6 mm i.d., 5 µm d_p) was purchased from Nacalai USA, Inc. (San Diego, CA, USA) and a bare silica RXSIL column (15 cm x 4.6 mm i.d., 3 µm d_p) was obtained from Agilent Technologies (Brussel, Belgium). High purity liquid carbon dioxide was obtained from Air Liquide (Herenthout, Belgium).

2.2. Instrumentation

A Mettler-Toledo TGA (Model SDTA851e, Mettler-Toledo, Zaventem, Belgium) controlled by STAR software provided with the instrument (version SW 9.00) was utilized for the thermogravimetric analysis of the synthesized stationary phases. The conditions were as follows: approximately 3 mg of sample was weighed out and the temperature range was from 25 – 1100°C at 10°C/min.

The SFC system used for LSER characterization was described elsewhere[18]. The conditions were as follows: injection was 1 µL, the CO_2-methanol 90:10 (v/v) mobile phase flow rate was isocratic at 3 mL/min, the outlet pressure was 150 bar, detection wavelength was 254 nm, and the temperature was isothermal at 25°C. These conditions were chosen to match the conditions used to establish a column classification for SFC[18] so as to provide comparison points.

Table 1. Supplement: List of compounds used for LSER characterization.

No	Compound	E	S	A	B	V
1	Benzene	0.610	0.52	0.00	0.14	0.7164
2	Toluene	0.601	0.52	0.00	0.14	0.8573
3	Ethylbenzene	0.613	0.51	0.00	0.15	0.9982
4	Propylbenzene	0.604	0.50	0.00	0.15	1.1391
5	Butylbenzene	0.600	0.51	0.00	0.15	1.2800
6	Pentylbenzene	0.594	0.51	0.00	0.15	1.4209
7	Hexylbenzene	0.591	0.50	0.00	0.15	1.5620
8	Heptylbenzene	0.577	0.48	0.00	0.15	1.7029
9	Octylbenzene	0.579	0.48	0.00	0.15	1.8438
10	Nonylbenzene	0.578	0.48	0.00	0.15	1.9847
11	Decylbenzène	0.579	0.47	0.00	0.15	2.1256
12	Undecylbenzene	0.579	0.47	0.00	0.15	2.2665
13	Dodecylbenzene	0.571	0.47	0.00	0.15	2.4074
14	Tridecylbenzene	0.570	0.47	0.00	0.15	2.5483
15	Tetradecylbenzene	0.570	0.47	0.00	0.15	2.6892
16	Allylbenzene	0.717	0.60	0.00	0.22	1.0961
17	Cumene	0.602	0.49	0.00	0.16	1.1391
18	*t*-Butylbenzene	0.614	0.49	0.00	0.16	1.28
19	*o*-Xylene	0.663	0.56	0.00	0.16	0.9980
20	*m*-Xylene	0.623	0.52	0.00	0.16	0.9980
21	*p*-Xylene	0.613	0.52	0.00	0.16	0.9980
22	Naphthalene	1.340	0.92	0.00	0.20	1.0854
23	1-Methylnaphthalene	1.344	0.90	0.00	0.20	1.2260
24	2-Methylnaphthalene	1.304	0.92	0.00	0.20	1.2260
25	1-Ethylnaphthalene	1.371	0.87	0.00	0.20	1.3670
26	2-Ethylnaphthalene	1.331	0.87	0.00	0.20	1.3670
27	Aniline	0.955	0.96	0.26	0.50	0.8162
28	N,N-Dimethylaniline	0.957	0.84	0.00	0.47	1.0980
29	Pyridine	0.631	0.84	0.00	0.52	0.6753
30	2-Ethylpyridine	0.613	0.70	0.00	0.49	0.9570
31	Phenylurea	1.110	1.40	0.77	0.77	1.0730
32	Caffeine	1.500	1.60	0.00	1.35	1.3630
33	Nicotinamide	1.010	1.09	0.63	1.00	0.9317
34	Indazole	1.180	1.25	0.54	0.34	0.9050
35	Carbazole	1.787	1.42	0.47	0.26	1.3150
36	Acridine	2.356	1.32	0.00	0.58	1.4130
37	*o*-Toluidine	0.966	0.92	0.23	0.45	0.957
38	*m*-Toluidine	0.946	0.95	0.23	0.55	0.957
39	*p*-Toluidine	0.923	0.95	0.23	0.52	0.957
40	Naphtylamine	1.670	1.26	0.20	0.57	1.1850
41	Benzoic acid	0.730	0.90	0.59	0.40	0.9317
42	Naphtoic acid	1.200	1.27	0.52	0.48	1.3007
43	Naphtylacetic acid	1.300	1.35	0.54	0.40	1.3007

Table 1. Supplement: List of compounds used for LSER characterization.

No	Compound	E	S	A	B	V
44	Anisole	0.708	0.75	0.00	0.29	0.9160
45	Phenylethanol	0.784	0.83	0.30	0.66	1.0570
46	Benzyl alcohol	0.803	0.87	0.39	0.56	0.9160
47	Benzaldehyde	0.820	1.00	0.00	0.39	0.8730
48	Acetophenone	0.818	1.01	0.00	0.48	1.0139
49	Propiophenone	0.804	0.85	0.00	0.51	1.1548
50	Valerophenone	0.795	0.95	0.00	0.50	1.4366
51	Coumarine	1.060	1.79	0.00	0.46	1.0620
52	Benzonitrile	0.742	1.11	0.00	0.33	0.8711
53	Nitrobenzene	0.871	1.11	0.00	0.28	0.8906
54	Chlorobenzene	0.718	0.65	0.00	0.07	0.8288
55	Bromobenzene	0.882	0.73	0.00	0.09	0.8910
56	Iodobenzene	1.188	0.82	0.00	0.12	0.9750
57	*o*-Methylacetophenone	0.780	1.00	0.00	0.51	1.1550
58	*m*-Methylacetophenone	0.806	1.00	0.00	0.51	1.1550
59	*p*-Methylacetophenone	0.842	1.00	0.00	0.52	1.1550
60	Benzophenone	1.447	1.50	0.00	0.50	1.4810
61	Naphthalene methanol	1.640	1.19	0.27	0.64	1.2850
62	Naphthalene ethanol	1.670	1.21	0.23	0.72	1.4259
63	Naphtylaldehyde	1.470	1.19	0.00	0.47	1.2420
64	Naphtylacetate	1.130	1.25	0.00	0.62	1.4416
65	Cyanonaphthalene	1.190	1.25	0.00	0.41	1.2401
66	Naphtylacetonitrile	1.430	1.44	0.00	0.53	1.3810
67	Nitronaphthalene	1.600	1.51	0.00	0.29	1.2596
68	Fluoronaphthalene	1.320	0.82	0.00	0.18	1.1030
69	Chloronaphthalene	1.540	0.92	0.00	0.15	1.2078
70	Bromonaphthalene	1.670	0.97	0.00	0.17	1.2604
71	Iodonaphthalene	1.840	1.04	0.00	0.20	1.3436
72	Phenol	0.805	0.89	0.60	0.30	0.7751
73	Eugenol	0.946	0.99	0.22	0.51	1.3540
74	Vanillin	1.040	1.04	0.32	0.67	1.1313
75	Resorcinol	0.980	1.00	1.10	0.58	0.8340
76	α-Naphtol	1.520	1.05	0.61	0.37	1.1441
77	β-Naphtol	1.520	1.08	0.61	0.40	1.1440
78	*o*-Cresol	0.840	0.86	0.52	0.30	0.9160
79	*m*-Cresol	0.822	0.88	0.57	0.34	0.9160
80	*p*-Cresol	0.820	0.87	0.57	0.31	0.9160
81	2,3-Dimethylphenol	0.850	0.85	0.52	0.36	1.0569
82	2,4-Dimethylphenol	0.840	0.80	0.53	0.39	1.0570
83	2,5-Dimethylphenol	0.840	0.79	0.54	0.37	1.0570
84	2,6-Dimethylphenol	0.860	0.79	0.39	0.39	1.0570
85	3,4-Dimethylphenol	0.830	0.86	0.56	0.39	1.0570
86	3,5-Dimethylphenol	0.820	0.84	0.57	0.36	1.0570

Table 1. Supplement: List of compounds used for LSER characterization.

No	Compound	E	S	A	B	V
87	*o*-Isopropylphenol	0.822	0.79	0.52	0.44	1.1978
88	*m*-Isopropylphenol	0.811	0.92	0.55	0.46	1.1978
89	*p*-Isopropylphenol	0.791	0.89	0.55	0.38	1.1978
90	*o*-Chlorophenol	0.853	0.88	0.32	0.31	0.8975
91	*m*-Chlorophenol	0.909	1.06	0.69	0.15	0.8975
92	*p*-Chlorophenol	0.915	1.08	0.67	0.20	0.8975
93	*o*-Nitrophenol	1.045	1.05	0.05	0.37	0.9490
94	*m*-Nitrophenol	1.050	1.57	0.79	0.23	0.9490
95	*p*-Nitrophenol	1.070	1.72	0.82	0.26	0.9490
96	*o*-Nitrobenzylalcohol	1.059	1.11	0.45	0.65	1.0900
97	*m*-Nitrobenzylalcohol	1.064	1.35	0.44	0.64	1.0900
98	*p*-Nitrobenzylalcohol	1.064	1.39	0.44	0.62	1.0900
99	*o*-Nitrotoluene	0.866	1.11	0.00	0.28	1.0320
100	*m*-Nitrotoluene	0.874	1.10	0.00	0.25	1.0320
101	*p*-Nitrotoluene	0.870	1.11	0.00	0.28	1.0320
102	Methylbenzoate	0.733	0.85	0.00	0.48	1.0726
103	Ethylbenzoate	0.689	0.85	0.00	0.46	1.2140
104	Propylbenzoate	0.675	0.80	0.00	0.46	1.3540
105	Butylbenzoate	0.668	0.80	0.00	0.46	1.4953
106	Benzylbenzoate	1.264	1.42	0.00	0.51	1.6804
107	Dimethylphthalate	0.780	1.41	0.00	0.88	1.4290
108	Diethylphthalate	0.729	1.40	0.00	0.88	1.7110
109	Dipropylphthalate	0.713	1.40	0.00	0.86	1.9924
110	Dibutylphthalate	0.700	1.40	0.00	0.86	2.2700
111	Biphenyl	1.360	0.99	0.00	0.26	1.3242
112	Phenyl-naphthalene	1.910	1.08	0.00	0.30	1.6932
113	Acenaphthene	1.604	1.05	0.00	0.22	1.1726
114	Acenaphtylene	1.750	1.14	0.00	0.26	1.2156
115	Fluorene	1.588	1.03	0.00	0.20	1.3570
116	Phenanthrene	2.055	1.29	0.00	0.26	1.4540
117	Anthracene	2.290	1.34	0.00	0.26	1.4540
118	9-Methylanthracene	2.290	1.30	0.00	0.26	1.5950
119	Fluoranthene	2.377	1.53	0.00	0.20	1.5850
120	Pyrene	2.808	1.71	0.00	0.29	1.5850
121	Chrysene	3.027	1.73	0.00	0.36	1.8230
122	Benzo[a]pyrene	3.625	1.98	0.00	0.44	1.9536
123	Perylene	3.256	1.76	0.00	0.42	1.9536
124	Binaphthyl	2.820	1.81	0.00	0.31	2.0622
125	Triphenylene	3.000	1.71	0.00	0.42	1.8234
126	o-Terphenyl	2.194	1.61	0.00	0.38	1.9320
127	p-Terphenyl	2.194	1.61	0.00	0.38	1.9320

E: Excess molar refraction; S: Dipolarity/polarizability; A: Hydrogen bond acidity; B: Hydrogen bond basicity (McGowan's characteristic); V: Volume.

Other analyses were performed on a SFC system composed of a Sandra/Selerity 5000 SFC pump (Selerity Technologies, Salt Lake City, UT, USA), an Agilent 1200 HPLC system equipped with a high pressure cell and variable wavelength detector (Agilent Technologies, Brussels, Belgium), a Gerstel Multipurpose Sampler (MPS-3) autosampler (Mülheim an der Ruhr, Germany), and a Polaratherm column oven with mobile phase pre-heater (Selerity Technologies). SFC separation conditions are outlined in references[20,21] and in the text. An Agilent 1969A Orthogonal TOF mass spectrometer with APCI source (Agilent Technologies) was utilized for the analysis of the metoclopromide sample. The conditions for the source were as follows: capillary voltage 3000 V, source temperature 350°C, vaporization temperature 350°C, corona current 7 mA, flow rate of the nebulizer gas (N_2) 7 L/min and nebulizer pressure 50 psi.

2.3. Data analysis

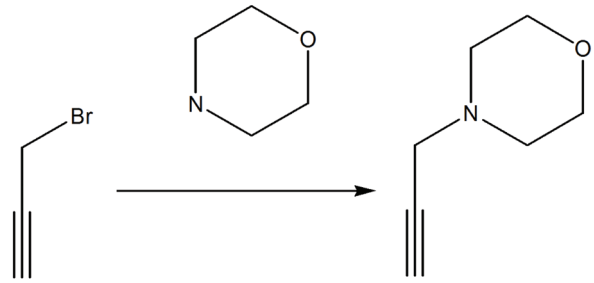
The LSER coefficients were obtained by multiple linear regression analysis on the logarithm of the measured retention factors (log *k*), carried out with XLSTAT 7.5 software (Addinsoft, New York, NY, USA). As recommended by recent reviews[22], the quality of the fits was estimated using the multiple correlation coefficient (R^2), adjusted determination coefficient (R^2_{adj}), standard error in the estimate (*SE*) and Fischer *F* statistic.

2.4. Synthesis of the stationary phases

The synthesis is based on the procedures described previously for estradiol and testosterone *click* stationary phases[14].

2.4.1. Morpholine precursor

The procedure by Verron et al.[23] was followed, though scaled-down, for the synthesis of morpholine precursor 4-prop-2-ynyl-morpholine (Scheme 2).



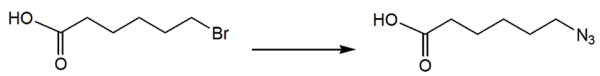
Scheme 2. Synthesis of 4-prop-2-ynyl-morpholine.

Morpholine (25 mL, 29 mmol) was dissolved in MeOH (250 mL) while cooled in ice and under an inert atmosphere. Once dissolved, potassium carbonate (21.9 g, 16 mmol) and propargyl bromide (31 mL, 29 mmol) were added with stirring while cooling was maintained. Stirring without cooling was continued for 4 h. The precipitate was filtered and washed with MeOH. The MeOH was removed by rotavap at 40°C. The precipitate was filtered using DCM (sonication was required to remove the precipitate from the round bottom flask). The DCM was then removed via rotavap at 40°C. The yellow oil remaining after completion was stored at 4°C. A percent yield of 83% was obtained.

GC-MS and NMR analysis were performed on the oil. The GC-MS chromatogram contained a single peak with only the final product present. No starting material was observed. The ^{1}H NMR shifts matched those reported by Verron et al.[23]. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.27 (d, 1H, CCH), δ = 2.57 (t, 4H, CH_2N), δ = 3.29 (d, 2H, $CCCH_2$), δ = 3.75 (t, 4H, CH_2O). ^{13}C NMR (300 MHz, $CDCl_3$): δ = 47.19 (s, 1C, CCH_2N), δ = 52.19 (s, 2C, CH_2N), δ = 66.84 (s, 2C, CH_2O), δ = 73.35 (s, 1C, $CCCH_2$), δ = 78.46 (s, 1C, $CCCH_2$).

2.4.2. Synthesis of 6-azidohexanoic acid.

6-azidohexanoic acid was synthesized according to Scheme 3.



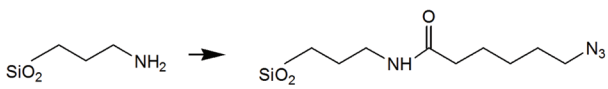
Scheme 3. Synthesis of 6-azidohexanoic acid.

Sodium azide (4.00 g, 62 mmol) and 6-bromohexanoic acid (6.00 g, 31 mmol) were combined in a reaction vessel with 40 mL DMF. A condenser was placed atop the reaction vessel and the reaction was stirred in an oil bath at 85°C overnight.

A liquid-liquid extraction was carried out in a separation funnel. DCM was used to extract out the product 6-azidohexanoic acid. The DCM extraction was carried out three times, and the DCM layers from each extraction were combined. Then 0.1 M HCl was used to extract the DMF from the collected DCM layers. This extraction was also carried out three times, and the DCM layers were collected. Magnesium sulfate was added to the DCM layer with stirring to dry the solvent. This was then filtered, and the DCM layer containing the final product was rotavaped at 40°C. A yellow oil was obtained. This synthesis was also performed in a scaled-up reaction. The average percent yield obtained was 72%.

^{1}H NMR and IR analyses were performed on the oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.45 (m, 2H, $CH_2(CH_2)_2$), δ = 1.67 (m, 4H, $CH_2(CH_2)_2$), δ = 2.38 (t, 2H, CH_2CO), δ = 3.29 (m, 2H, CH_2N_3). The IR spectra contained a band at 2093 cm^{-1}, which corresponds to the presence of an azide.

2.4.3. Attachment of 6-azidohexanoic acid to the aminopropyl silica particles (Scheme 4)

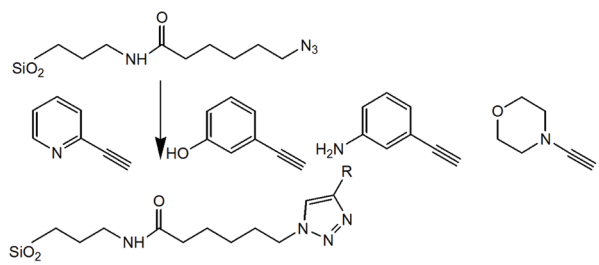


Scheme 4. Attachment of 6-azidohexanoic acid to aminopropyl silica particles.

Based on the amine loading (C 4.4%, N 1.3%) of the silica particles used, the ratio was 1:3:3:6 amino groups: 6-azidohexanoic acid: PyBOP: DPI. Thus, aminopropyl silica (10 g, 12 mmol amine), 6-azidohexanoic acid (5.65 g, 36 mmol), PyBOP (18.70 g, 36 mmol), and DPI (12.5 mL, 72 mmol) were all combined in 144 mL DMF. This reaction was shaken at room temperature for 4.5 h. The particles were filtered using a size 4 filter. The particles were washed three times each with DMF, MeOH, and DCM. The particles were then allowed to dry in an oven at 60°C before endcapping occurred. To endcap, the entire product from the above reaction was combined with 10 mL pyridine, 30 mL acetic acid anhydride, and 60 mL DMF. This reaction was shaken at room temperature for 1 h. The particles were filtered using a size 4 filter. The particles were washed three times each with DMF, MeOH, and DCM, and then dried in an oven at 60°C.

A color test was performed to determine if free amines were still present[24,25]. To the endcapped aminopropyl silica modified with 6-azidohexanoic acid, 3 drops of picrylsulfonic acid (TNBS) solution and 5 drops of 10% DIEA/DMF solution were added. The picrylsulfonic acid will only react with free amines, giving a color (red) change. No color change was observed.

2.4.4. Clicking stationary phases to the azide-modified particles (Scheme 5)



Scheme 5. *Clicking* pyridine, phenol, aniline and morpholine.

All of the particles from the endcapping step were utilized for the *click* reaction: 4-prop-2-ynyl morpholine (7.5 g, 60 mmol), copper sulfate pentahydrate (3.0 g, 12 mmol), and sodium ascorbate (12.0 g, 60 mmol) were combined with the particles in 240 mL 1:1 water:isopropanol. This material is referred to as *clicked* morpholine (C-Mor). For the other stationary phases, 2-ethynyl pyridine (6.1 g, 60 mmol) was used for the *clicked* pyridine (C-Pyr) phase, 3-ethynyl phenol (6.9 g,

60 mmol) for the *clicked* phenol (C-Phe) phase, and 3-ethynyl aniline (7.1 g, 60 mmol) for the *clicked* aniline phase (C-Anal). All other conditions were the same as for the C-Mor phase. After 24 h, the particles were filtered with a size 4 filter and washed three times each with DMF, MeOH, and DCM. However, the particles still contained Cu^{2+} from the catalyst used in the *click* reaction. Therefore, the particles were suspended in a 0.1 M EDTA solution. This was allowed to shake overnight at room temperature. The particles were then filtered and washed three times each with water, MeOH, and DCM. The particles were then allowed to dry at 60°C for 1 h. The synthesized stationary phases (i.e.C-Pyr, C-Phe, C-Anil and C-Mor) were packed into 15 cm x 3 mm i.d. columns by Mel Euerby from HiChrom (Berkshire, UK).

3. Results and discussion

3.1. Thermogravimetric analysis (TGA)

Thermogravimetric analysis (TGA) was performed to determine the load of the *click* reactions on the aminopropyl silica. All phases show an increase with ca. 10% compared to the aminopropyl silica as such (Figure 1).

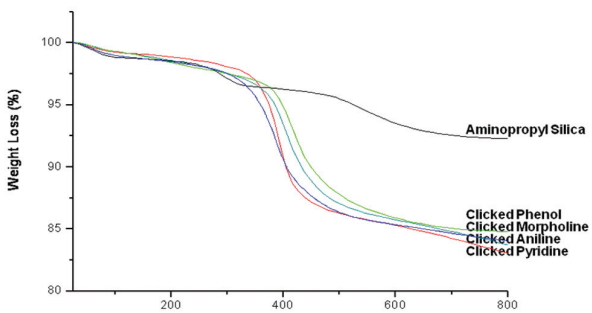


Figure 1. Thermogravimetric analysis (TGA) plots of the synthesized *click* stationary phases.

3.2. LSER characterization

The QSRR (quantitative structure-retention relationship) approach furnishes a detailed and reliable description of the role and extent of the different molecular interactions that can be established between the analytes and the chromatographic system. Among QSRRs, the solvation parameter model using Abraham descriptors has gained acceptance as a general tool to explore the factors affecting retention in chromatographic systems[20]. The retention of selected probes in a dense fluid can be related through this relationship, also known as linear solvation energy relationship (LSER), to specific interactions by the following equation:

$$\log k = c + eE + sS + aA + bB + vV \quad (1)$$

In this equation, capital letters represent the solute descriptors, related to particular interaction properties, while lower case letters represent the system constants, related to the complementary effect of the phases on these interactions. *c* is the model intercept term, which when the retention factor is used as the dependent variable is dominated by the phase ratio. E is the excess molar refraction (calculated from the refractive index of the molecule) and models polarizability contributions from *n* and ϖ electrons; S is the solute dipolarity / polarizability; A and B are the solute overall hydrogen-bond acidity and basicity; V is the McGowan characteristic volume in units of $cm^3\ mol^{-1}/100$. The system constants (*e*, *s*, *a*, *b*, *v*), obtained through a multilinear regression of the retention data for a certain number of solutes with known descriptors, reflect the magnitude of difference for that particular property between the mobile and stationary phases. Thus, if a particular coefficient is numerically large, then any solute having the complementary property will interact very strongly with either the mobile phase (if the coefficient is negative) or the stationary phase (if the coefficient is positive). Eq. (2) can be deduced from Eq. (1):

$$\log\alpha = e\Delta E + s\Delta S + a\Delta A + b\Delta B + v\Delta V \quad (2)$$

where α is the separation factor between two solutes and ΔX represents the difference in the X coefficient between these two solutes. Consequently, the coefficients also reflect the system's selectivity towards any particular molecular interaction.

Moreover, characterising different stationary phases while always using the same mobile phase and

operating conditions ensures that the LSER coefficients can be compared to provide a comparison of the stationary phase properties. Numerous stationary phases have been evaluated with this method in SFC conditions[18,19], to provide a classification of stationary phases for pSFC uses.

Furthermore, based on[26], the angle between two solvation vectors (ω) associated to two chromatographic systems can be calculated according to the following equation, based on the solvation parameter model coefficients of the two systems noted i and j:

$$\cos\theta_{ij} = \frac{\vec{\omega}_i * \vec{\omega}_j}{|\vec{\omega}_i| * |\vec{\omega}_j|} = \frac{e_i e_j + s_i s_j + a_i a_j + b_i b_j + v_i v_j}{\sqrt{e_i^2 + s_i^2 + a_i^2 + b_i^2 + v_i^2}\sqrt{e_j^2 + s_j^2 + a_j^2 + b_j^2 + v_j^2}} \quad (3)$$

The angle between two columns provides a mean to measure the informational equivalence of different chromatographic systems. However, this information is not sufficient to judge whether two stationary phases are similar, as it does not take into account the confidence limits associated to the system constants.

The similarity between two chromatographic systems is thus evaluated through the calculation of the J similarity factor, determined through Eqs. (4), (5) and (6):

$$J = \cos\theta_{ij} - \cos(\theta_{di} + \theta_{dj}) \quad (4)$$

$$\cos(\theta_{di} + \theta_{dj}) = \sqrt{\left(1 - \frac{D_i^2}{|\vec{\omega}_i|^2}\right)\left(1 - \frac{D_j^2}{|\vec{\omega}_j|^2}\right)} - \frac{D_i D_j}{|\vec{\omega}_i||\vec{\omega}_j|} \quad (5)$$

$$D = TINV(1 - 0.99, N) \bullet SE \quad (6)$$

where TINV is the inverse of the Student's t-distribution for the specified degrees of freedom N, and SE is the average of the standard errors of the solvation parameter model coefficients.

In Eq. (4), when J is positive, the systems compared are found to be similar; in the opposite case, they are considered to be different.

When two stationary phases are similar, it indicates that the elution order of analytes will be very similar on the two chromatographic systems. However, retention might be different. The global intensity of the interactions can be compared through the values of the solvation vector length, calculated as follows:

$$u_i = \sqrt{e_i^2 + s_i^2 + a_i^2 + b_i^2 + v_i^2} \quad (7)$$

Thus when u_i and u_j are close, retention will be similar on both phases (provided phase ratio is close), while different values of vector length indicates that retention and separation factors will be larger on the chromatographic system providing larger values of *u*.

The results obtained with the commercially available 2-ethyl pyridine column, 1,2,4-triazole column and bare silica column were compared to those obtained with the *clicked* columns. The 2-ethyl pyridine column was chosen for the purpose of comparison because one of the *click* stationary phases has a pyridine ring as a terminal function, and because it is a very popular column among SFC users. The 1,2,4-triazole column was also an interesting comparison point because it has a triazole ring as the stationary phase, whereas with the *clicked* columns, a triazole ring is embedded in the linker. Note, however, that the comparison cannot be completely correct as the 1,2,3-triazole ring in the *click* phases has a pKa of 1.2 while the 1,2,4-triazole ring has a pKa of 2.2.

All the models calculated for the six stationary phases (the four *click* stationary phases, and the 2-ethyl pyridine and 1,2,4-triazole phase) in this study are presented in Table 2, together with the statistics of the regression equations.

The fits for the click stationary phases were all of reasonable quality, R^2_{adj} ranging from 0.933 to 0.964, standard error in the estimate varying from 0.109 to 0.147. All coefficients are significantly larger than their standard deviation; therefore, the results are amenable to interpretation. Only few outliers were eliminated from the models but, in any case, always more than

Table 2. System constants and model fit statistics.

Stationary phase	*c*	*e*	*s*	*a*	*b*	*v*	n	R^2	R^2_{adj}	*SE*	*F*	*u*
C-Phenol	-1.245	0.604	0.208	1.222	0.749	-0.247	115	0.936	0.933	0.145	319	1.59
	0.069	*0.038*	*0.071*	*0.060*	*0.081*	*0.054*						
C-Aniline	-1.340	0.540	0.426	1.506	0.409	-0.143	108	0.966	0.964	0.109	573	1.71
	0.056	*0.031*	*0.063*	*0.048*	*0.068*	*0.041*						
C-Morpholine	-1.155	0.537	0.214	1.654	0.359	-0.254	117	0.939	0.937	0.147	343	1.81
	0.067	*0.038*	*0.072*	*0.060*	*0.081*	*0.052*						
C-Pyridine	-1.345	0.528	0.247	1.648	0.298		113	0.949	0.947	0.131	506	1.77
	0.041	*0.033*	*0.064*	*0.049*	*0.072*							
2-ethyl pyridine	-1.057	0.588	0.564	1.053	0.790	-0.692	120	0.929	0.926	0.191	297	1.70
	0.086	*0.050*	*0.094*	*0.069*	*0.120*	*0.071*						
1,2,4-triazole	-1.244	0.595		1.556	1.037	-0.491	100	0.934	0.932	0.136	339	2.02
	0.067	*0.031*		*0.058*	*0.086*	*0.068*						

n is the number of solutes considered in the regression, R^2 is the multiple correlation coefficient, R^2_{adj} is the adjusted correlation coefficient, *SE* in the standard estimate error, *F* is Fischer's statistic and the numbers in italics represent 99% confidence limits, is the length of the vector associated to the chromatographic system, calculated according to equation (7).

100 compounds were retained in the final model calculation, and the diversity of chemical functions is unaltered by the removal of outliers, thus all conclusions should be highly substantial.

Using the solvation parameter model, the different chromatographic systems can be compared based on the values of the regression coefficients. First of all, it can be noticed that all polar-type interactions (*e*, *s*, *a*, *b*) are positive, indicating that they are stronger between analytes and stationary phase than between analytes and mobile phase, while the dispersive interactions (*v*) are negative or zero. This pattern is classical with polar stationary phases characterized in supercritical mobile phases: an increase in polarity of the analytes causes an increase in retention, while an increase in hydrocarbon volume causes a decrease in retention.

The *a* coefficient is the largest on all columns, indicating significant basic character of the stationary phases. Second most important coefficients are *b* (indicating acidic character of the stationary phase) or *e*, due to the aromatic character of all stationary phases.

The θ_{ij} angles existing between the solvation vectors associated to all the stationary phases characterized above through the use of the solvation parameter model and the *J* similarity factors at the 99% confidence limit between them were calculated, according to Eqs. (3) to (6). They can be observed in Table 3.

Based on angle calculation, C-Pyr cannot be discriminated from C-Mor. They display a 8° angle between them with slightly larger *u* vector length for C-Mor. This is also visible on the κ-κ plot (Figure 2A). The slope is close to 1, indicating generally similar interaction strength on the two phases. The intercept is positive indicating larger retention on C-Pyr than on C-Mor. The position of the regression line above the first bisector can possibly be related to a larger *v* coefficient. This is possibly due to the C-Pyr phase being somewhat less cohesive than the others, as will be discussed further below. In this case, solutes penetrate more easily in the stationary phase and are more retained.

The 2-ethyl pyridine stationary phase is significantly different from the *click* stationary phases, and in particular, it is very different from the C-Pyr stationary phase, with a measured angle of 36° between them. The κ-κ plot comparing the two pyridine-type phases (Figure 2B) indeed shows important differences between the two columns.

Table 3. Values of the θ angles between the solvation vectors.

θ_{ij}	C-Phenol	C-Aniline	C-Morpholine	C-Pyridine	2-ethyl pyridine	1,2,4-triazole
C-Phenol		17	18	22	21	11
C-Aniline	17		10	16	22	22
C-Morpholine	18	10		**8***	31	22
C-Pyridine	22	16	**8***		36	27
2-ethyl pyridine	21	22	31	36		23
1,2,4-triazole	11	22	22	27	23	

θ is calculated according to equation (3). The asterisks indicate the couples judged similar through the calculation of J, according to equations (4), (5) and (6), at the 99% confidence limit.

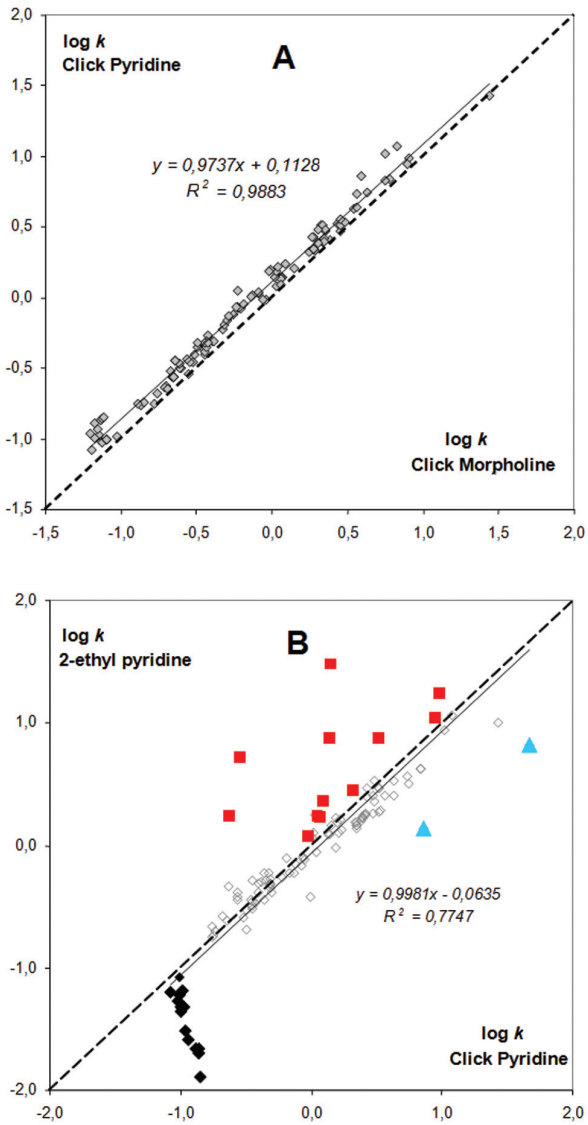


Figure 2. Comparison of the retention factors (log *k* – log *k* plots) for all compounds in Table 1 between (A) *click* Pyridine and *click* Morpholine and (B) 2-ethyl pyridine and *click* Pyridine. Black diamonds are alkylbenzenes (compounds 1 to 15 in Table 2); blue triangles are acidic solutes (n° 41 and 42 in Table 2); red squares are basic analytes (compounds 27 to 40 in Table 2).

Phase ratio seems quite close between the two columns, as most points are close to the first bisector, but three compound families exhibit different behaviours: (i) the alkylbenzene homologous series indicate that methylene selectivity is nearly zero on *click* Pyridine (which is consistent with a zero value of the *v* coefficient) while it is negative on 2-ethyl pyridine (in accordance with the significant negative *v* value); (ii) basic solutes are more retained on the 2-ethyl pyridine phase, as indicated by the largest *b* coefficient on 2-ethylpyridine and (iii) acidic solutes are more retained on the C-Pyr phase, as indicated by the largest *a* coefficient.

Judging from the angle values, the 1,2,4-triazole phase is also significantly different from the *click* phases, indicating significant contribution of the terminal ligand to retention on the *click* phases. However, the angles are smaller between *click* phases and 1,2,4-triazole than between *click* phases and 2-ethylpyridine. C-Pyr appears to be the closest to the 1,2,4-triazole phase, with 11° and 13° angles respectively.

Further information can be retrieved from a close examination of the LSER coefficients.

The *c* constant does not vary significantly between the five click columns and will thus not be discussed.

3.2.1. The e coefficient

This coefficient does not vary significantly among the six phases (bare silica is included for comparison, see further), as can be seen on Figure 3A. This is not

surprising as the *e* coefficient only shows very little variance among all stationary phases characterized in SFC[19]. This is partly due to its composite nature (it represents dispersive, ϖ-ϖ and dipole-induced dipole interactions), and partly due to the fact that all the test solutes (Table 1) possess an aromatic group (to allow for UV detection) thus the capability to interact through ϖ and non-binding electrons cannot be correctly assessed. However, as all the stationary phases in this study also have an aromatic group or at least double-bonds and non-binding electrons, it can be expected that, even with non-aromatic solutes in the test-set, no great differences would be seen.

3.2.2. The s coefficient

This coefficient varies to a greater extent (Figure 3B). The S descriptor is associated to heterogeneous charge repartition in the analyte structure, thus the *s* coefficient represents dipole-dipole type interactions. C-Anil, in particular, is seen to provide stronger dipole-dipole and dipole-induced dipole interactions than the others. However, the strongest dipole-dipole interactions are observed on the 2-ethyl pyridine phase. On the 1,2,4-triazole phase, and only on this one, the *s* coefficient is zero, indicating that the introduction of a terminal function clearly provides some differences as compared to the triazole ring alone.

3.2.3. The a coefficient

As mentioned above, this coefficient is the largest on all phases (Figure 3C). As it indicates retention of acidic solutes, it is related to the basic character of the stationary phase, or to its ability to participate in hydrogen-bond interactions as an electron-donor. C-Phe is clearly less basic than C-Anil, C-Mor and C-Pyr, as could have been expected, based on the nature of the ligand: indeed, the phenol functional groups are naturally less basic than the nitrogen atoms of the latter three. C-Mor and C-Pyr are nearly identical. C-Anil is close to C-Mor and C-Pyr. 1,2,4-Triazole is also among the most basic phases, while the 2-ethyl pyridine phase is significantly less basic than all others. This suggests that the 1,2,3-triazole ring is taking a significant part in the basic character of the *click* stationary phases.

3.2.4. The b coefficient

This coefficient provides a variety of interactions among the tested stationary phases (Figure 3D). As it indicates retention of basic solutes, it is related to the acidic character of the stationary phase, or to its ability to participate in hydrogen-bond interactions as a proton-donor. As expected based on stationary phase structure, C-Phe is more acidic than the "basic" phases, C-Anil, C-Mor and C-Pyr.

The 1,2,4-triazole phase is the most acidic of all. The exact nature of the stationary phase structure is unknown to us, as the information provided by the manufacturer only shows the triazole ring, without any details as regards possible spacer arms. Thus we have two possible explanations: either the –NH= group from the triazole ring or residual silanol groups can be responsible for extra retention of the basic solutes on this phase.

The 2-ethyl pyridine is also among the most acidic phases, which is surprising judging that the pyridine function is not acidic. Residual silanol groups or some other functional groups could be responsible for the strong acidic character.

3.2.5. The v coefficient

This coefficient also varies to a large extent (Figure 3E), especially for the 1,2,4-triazole and 2-ethyl pyridine phases, which display large negative *v* values, and for C-Pyr, the only phase where this coefficient is zero. As it is negative on polar phases, care must be taken in the interpretation of the *v* coefficient. When it increases (decreases in absolute value), it indicates a less cohesive phase and/or more dispersive interactions.

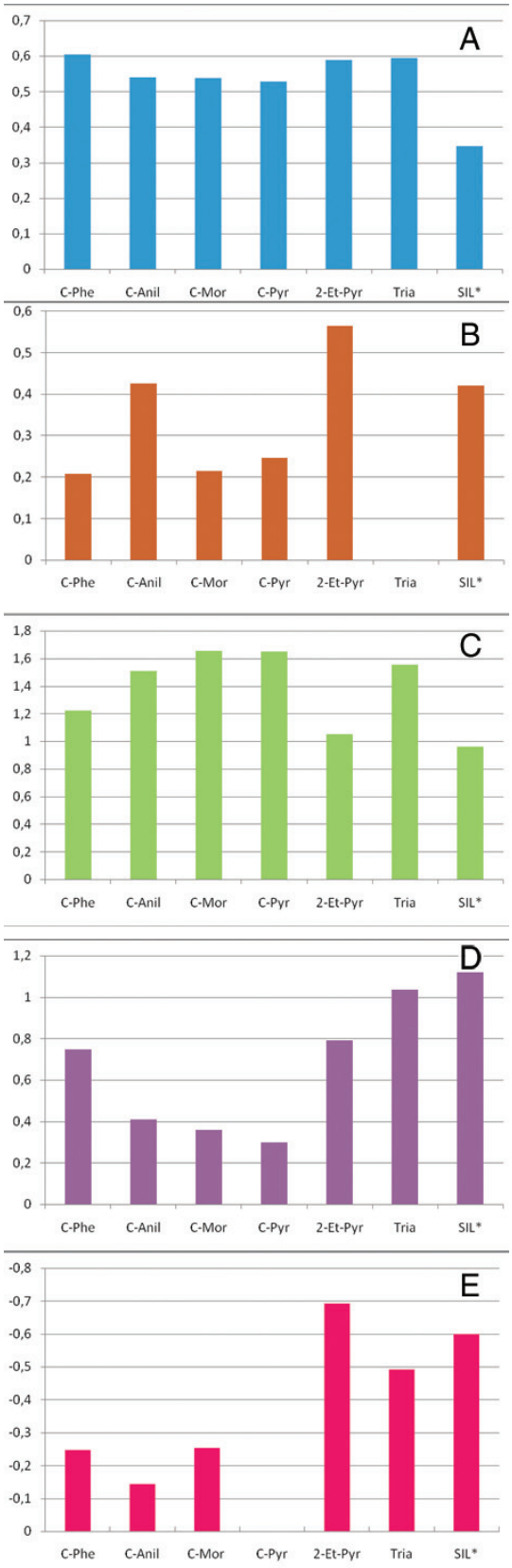


Figure 3. LSER coefficients calculated for the seven columns studied. *e* coefficient (A), *s* coefficient (B), *a* coefficient (C), *b* coefficient (D), *v* coefficient (E).

3.2.6. Systems constants for bare silica

The system constants for bare silica have been included in Figure 3 because in our opinion[20,21] and supported by recent literature on porous[27] and core shell silica particles[28], this stationary phase has unique characteristics for SFC. Moreover, its quality is presently such that acidic as well as basic solutes can be analysed by SFC without addition of additives (see further). Note in Figure 3 that silica compared to the other phases is the most proton donor (acidic) and the less proton acceptor (basic) phase.

3.3. Chromatographic analysis

The above detailed classification gives a general picture of the polarity/selectivity of the *click* phases compared to other polar, moderately polar aromatic, polar alkyl, and non-polar alkyl phases[17]. The *click* phases all belong to the polar phases in SFC or normal phases in LC. Upon injecting the 10 component standard mixture, all phases show a group separation of acids/bases from the neutral hydrophobic species. The latter elute at the end of the chromatogram and always in the same elution order i.e. cortisone, prednisone, hydrocortisone and prednisolone. A typical chromatogram is shown for the commercial 1,2,4-triazole column in Figure 4A. The same profile was obtained for the 2-ethyl pyridine, the C-Pyr and C-Mor phases. The C-Phe and C-Anil phases showed a different elution order for the acid and bases and a much more pronounced group separation for polar/neutrals. This is illustrated in Figure 4B for the analysis of the same mixture on the C-Phe column. This column showed excellent selectivity for samples of drug discovery and was experienced to have a broader applicability range compared to the C-Anil phase.

From the LSER characterization and also based on a large number of analyses of pharmaceutical samples, the C-Phe and C-Mor phases were considered most valuable for selectivity optimization for real samples.

As an example, the complementary nature of silica and C-Mor is illustrated for the analysis of an

acid/base mixture. On silica (Figure 5A), the acids elute before the bases and flurbiprofen/fenoprofen are not separated. On the C-Mor phase, the acid/base order is disturbed because the bases elute faster on this phase. For both chromatograms, no additives were used illustrating the high inertness of the phases. The same conclusion is valid for all phases evaluated in this study.

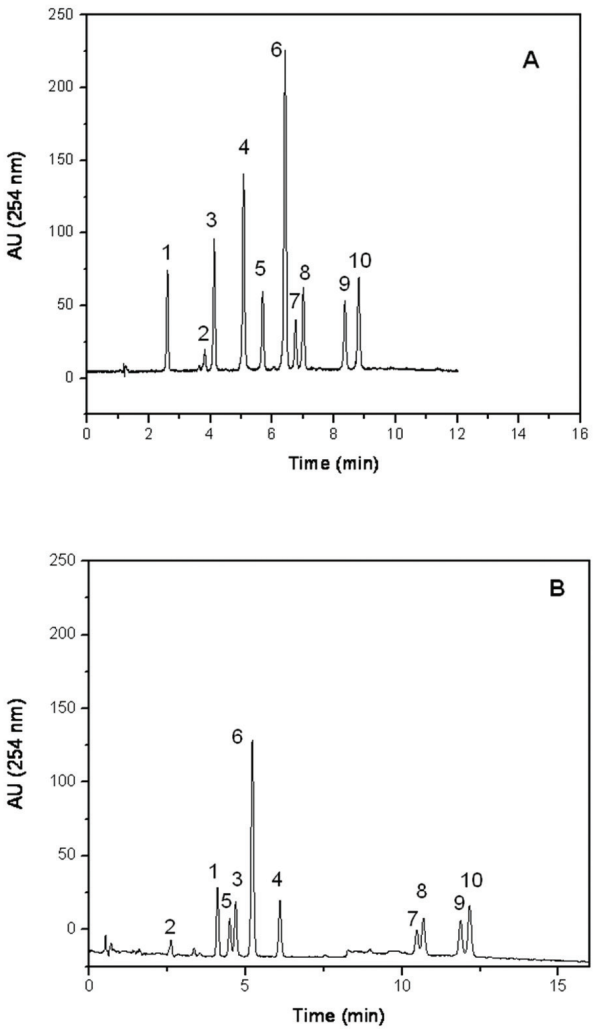


Figure 4. Chromatograms of the 10-component test mixture on the 1,2,4-triazole column (15 cm x 4.6 mm i.d., 5 µm d_p) and the *click* Phenol column (15 cm x 3 mm i.d., 5 µm d_p (B). The separation conditions were as follows: (A) Flow rate CO_2 2.0 mL/min, flow rate modifier from 0-20 min 0.12 to 1.0 mL/min (5.7 – 33% modifier). (B) Flow rate CO_2 = 1.6 mL/min, flow rate modifier from 0-20 min 0.1 to 0.8 mL/min (5.7 – 33% modifier). Modifier MeOH 20 mM ammoniumformate. Detection UV 254 nm. Temperature 40°C. Peaks: 1. caffeine, 2. ibuprofen, 3. theophylline, 4. theobromine, 5. fenoprofen, 6. flurbiprofen, 7. cortisone, 8. prednisone, 9. hydrocortisone, 10. prednisolone.

The C-Phe column was selected for the analysis of metoclopramide and its potential impurities by SFC-TOFMS operated in the positive APCI mode. Details on

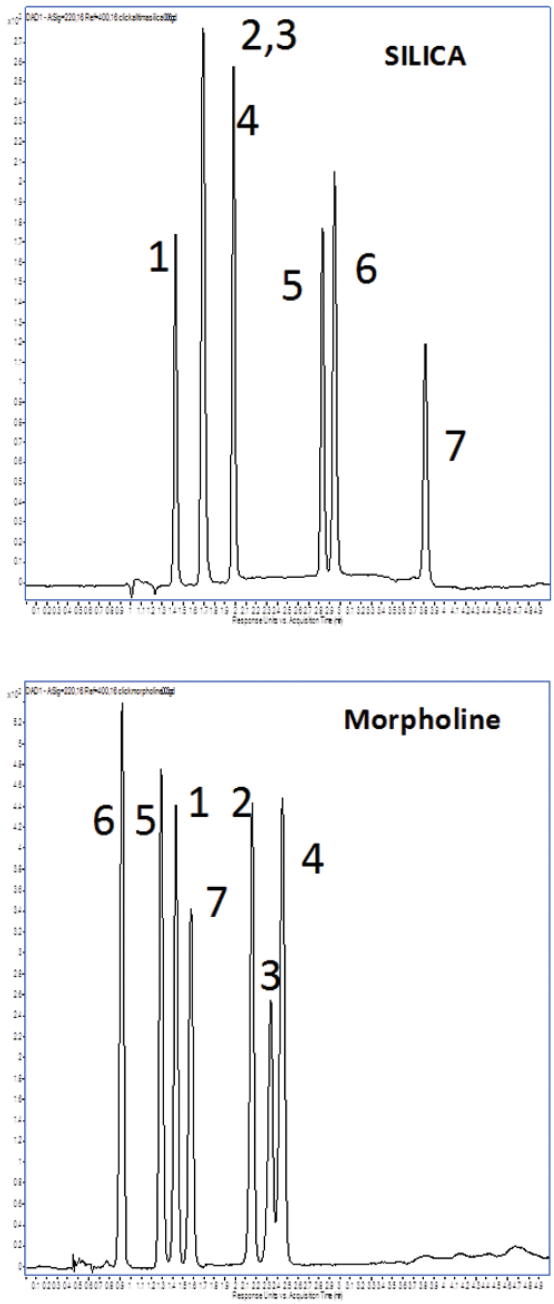


Figure 5. Chromatograms of the acid-base mixture on (A) bare silica (15 cm x 4.6 mm i.d., 3 µm d_p) and (B) *click* Morpholine (15 cm x 3 mm i.d., 5 µm d_p). The separation conditions were as follows: Flow rate CO_2 2.0 mL/min, flow rate modifier from 0-5 min 0.2 to 0.7 mL/min. Modifier MeOH. Detection = UV 254 nm. Temperature = 40°C. Peaks: 1. ibuprofen, 2. flurbiprofen, 3. fenoprofen, 4. ketoprofen, 5. theophylline, 6. caffeine, 7. theophyllinfe.

the coupling can be found in ref[20,21]. Metoclopramide is basic (pK_a 9.3), and the impurities contain a mixture of acidic and basic compounds (Table 4). To enhance ionization 20 mM ammonium formate was added to the mobile phases. Figure 6 shows the UV and MS trace for the active pharmaceutical ingredient with the impurities

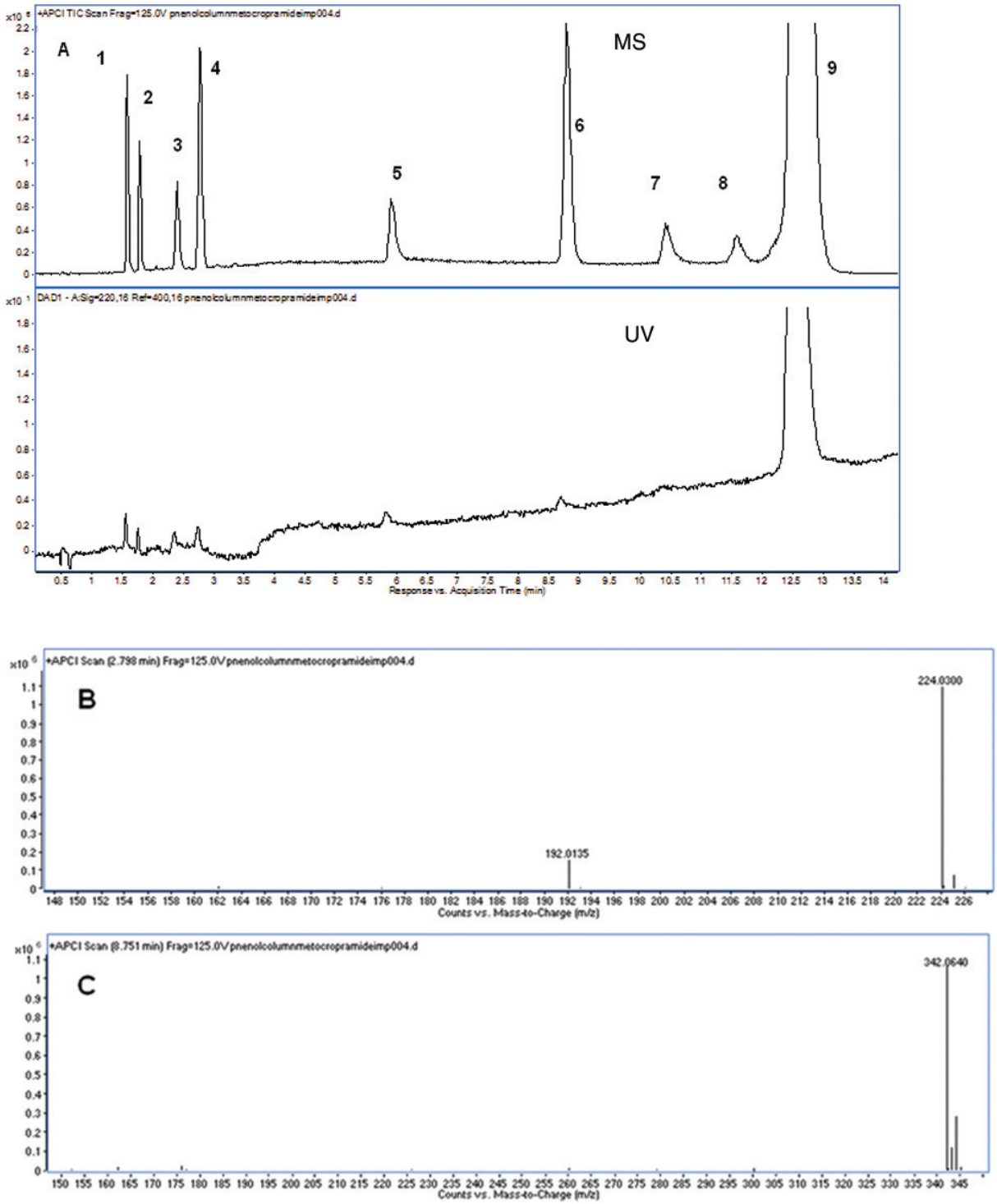
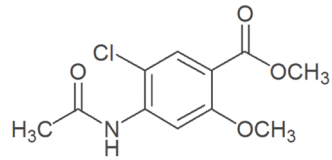
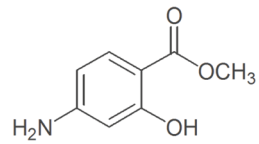
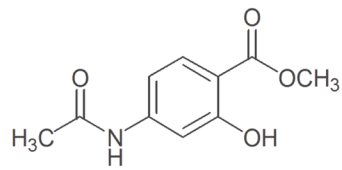
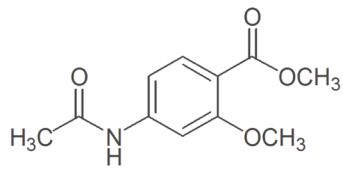
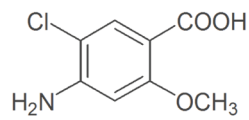
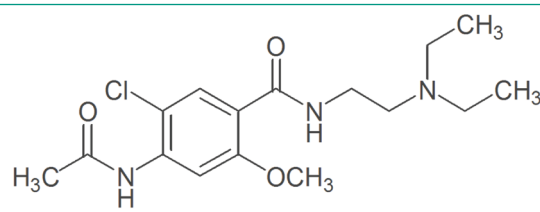
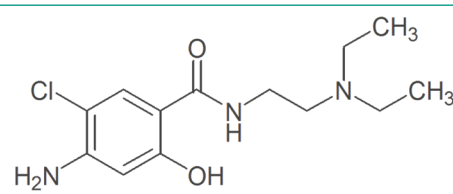
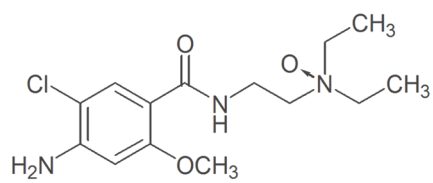
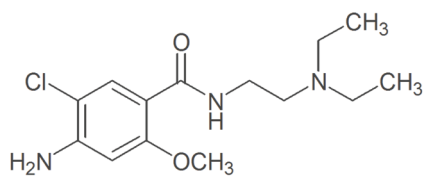


Figure 6. SFC-UV-TOFMS (A) of Metoclopramide and impurities at 0.02% level. Column: *click* Phenol column (15 cm x 3 mm i.d., 5 µm d_p).Separation Conditions: flow rate CO_2 1.6 mL/min, flow rate modifier from 0-21 min 0.1 to 1.2 mL/min. Modifier MeOH with 20 mM NH_4OOCH. UV 220 nm. MS scan. Temperature = 40°C. Outlet pressure 100 bar (fixed restrictor[28]). Injection 5 µL (metoclopramide 5 mg/mL, impurities at 0.02%). Mass spectra of peak 4 (B) and 6(C).

Table 4. Metoclopramide and impurities.

Peak	Name	Structure
1	Methyl 4-(acetylamino)-5-chloro-2-methoxybenzoate	
2	Methyl 4-amino-2-methoxybenzoate	
3	Methyl 4-(acetylamino)-2-hydroxybenzoate	
4	Methyl 4-(acetylamino)- 2-methoxybenzoate	
5	4-Amino-5-chloro-2-methoxybenzoic acid	
6	4-(Acetylamino)-5-chloro-N-2-(diethylaminoethyl)-2-methoxybenzamide	
7	4-Amino-5-chloro-N-2-(diethylaminoethyl)-2-hydroxybenzamide	
8	4-Amino-5-chloro-N-2-(diethylaminoethyl)-2-methoxybenzamide N-oxide	
9	Metoclopramide	

at the 0.02% level. In UV detection, only the 0.2% level could be quantified while the sensitivity in MS is much higher, easily allowing quantification at the 0.02% level. One of the advantages of SFC-APCI compared to reversed-phase LC-electrospray ionization (ESI) is that the spectra are very clean $[M+H]^+$ without formation of adducts typically observed in ESI. The mass spectra of impurity 4 and 6, both at 5 ng absolute on the column, are shown in Figure 6 C and D. Note also the excellent baseline in the scan SFC-MS trace illustrating that bleeding of the C-Phe is absent.

4. Conclusions

Stationary phases with different functionalities can easily be synthesized by the copper (I)-catalyzed azide-alkyne cycloaddition *click* reaction. Phenol, aniline, morpholine and pyridine were incorporated via a 1,2,3-triazole ring on aminopropyl silica. The C-Phe and C-Mor are complementary phases to the polar bare silica, 2-ethyl pyridine and 1,2,4-triazole. Based on the LSER studies, the main differences between the phases are related to the hydrogen-bonding capabilities (*a* and *b* coefficients), explaining the different elution orders observed between acidic and basic solutes. Additionally, it was shown that these *clicked* columns can be utilized for real-life applications. A pharmaceutical sample and impurities were analyzed using the C-Phe column with TOFMS detection in which 0.02% impurity levels were detected, which is sensitive enough for drug discovery.

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Analysis of Steroids using Solid Phase Microextraction-Gas Chromatography-Mass Spectrometry-Mass Spectrometry (SPME-GC-MS-MS)

Shilpi Chopra[1]
Paulo C. F. L. Gomes[2]
Ramkumar Dhandapani[1]
Nicholas H. Snow[1*]

[1]Department of Chemistry and Biochemistry, Center for Academic Industry Partneship, Seton Hall University, 400 South Orange Avenue, South Orange, NJ, 07079, USA
[2]Institute of Chemistry of Sao Carlos, University of Sao Paulo, Postal Code 780, 13560-970, São Carlos, SP, Brazil
*nicholas.snow@shu.edu

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Abstract

Direct immersion SPME-GC-MS-MS was used for the analysis of steroids in water at part-per-trillion(ppt) and lower concentrations. The method was validated and extended to real sample analysis. The method were linear from 0.01 to 5 ng/ml with precision less than 10% relative standard deviation for a steroid mixture at 1 ng/ml. Limit of quantitation and limit of detection was found to be 200- 1200 pg/L and 30-200 pg/L respectively and recoveries ranged from 88-103 %. To understand the extraction efficiency of the fiber, a depletion study was performed. The fiber/ sample partition coefficients for the steroids were determined to be 1.0×10^4 to 1.5×10^4. The extraction was performed without derivatization or the use of an internal standard. SPME-GC-MS-MS effectively demonstrated ultra-trace level detection of steroids in water.

Keywords: Steroids using Solid Phase Microextraction; SPME; Gas Chromatography-Mass Spectrometry-Mass Spectrometry; GC-MS-MS; SPME-GC-MS-MS; water analysis.

1. Introduction

Steroids are considered emerging contaminants in water and interest in their analysis in environmental water has increased over the past several years. The presence of human hormones in water systems was reported as early as 1965, suggesting that steroids are not entirely eliminated during wastewater treatment[1]. An association between a synthetic birth-control pharmaceutical (ethynylestradiol) and impact on fish was noted in the 1990's[2]. Even very low concentrations of steroids in water systems can lead to changes in the endocrine systems of aquatic life, which may result in effects on growth and development, decrease in fertility, feminiazation and hermaphroditism; these changes may be expressed in current and in future generations[3]. Sewage water treatment plants do not completely remove pharmaceuticals and hormones[4,5], resulting in contamination of water systems[6]. Steroidal hormones are excreted by both humans and livestock and are deposited into river systems through sewage treatment and agricultural runoff [7].

Steroids may reach groundwater by several means, including penetration through substrata, migration through agricultural soils amended with litter[8], migration from septic systems[9] or movement through unconsolidated river bed sediments[10]. Several analytical techniques are generally employed for the analysis of steroids in water, including liquid chromatography mass spectrometry[11], gas chromatography with ion trap mass spectrometry and tandem mass spectrometry[12], liquid chromatography-mass spectrometry with laser diode ionization[13], GCxGC-time of flight mass spectrometry[14] and tandem mass spectrometry[15]. Gas chromatography and liquid chromatography are the most commonly used techniques for the separation of steroid mixtures. Recently, multi-dimensional liquid chromatography[16,17] and multidimensional gas chromatography[18,19] are widely used as they add another dimension of selectivity to the separation.

The steroids used in this work are shown in Figure 1. Mesterolone and its metabolites have been isolated by liquid–liquid extraction and analyzed by gas chromatography–mass spectrometry (GC–MS) after acylation or silylation, with five metabolites detected[20]. Methandriol is an anabolic steroid derived from dihydotestosterone; high-sensitivity analysis of this and other female-steroid hormones in environmental samples which included estrone, estradiol and methandriol was performed using LC- tandem mass spectrometry with LOD's 0.1-3.1 ng/L[21,22]. Estrone is an aromatized C_{18} steroid with a 3-hydroxyl group and a 17-ketone, a major mammalian estrogen. 17-α-estradiol is the most potent mammalian estrogenic steroid. Etiocholanolone (or aetiocholanolone) is a metabolite of testosterone and has been analyzed in urine by GC-IR-MS[23]. Androstenedione is a steroid sex hormone that is secreted by the testes, ovaries, and adrenal cortex and is an intermediate in the biosynthesis of testosterone and estrogen. Prasterone an endogenous inactive compound by itself, is converted to estrogens and/or androgens in peripheral tissues which possess the required steroidogenic enzymes into cell-specific intracellular E2 and testosterone by the mechanisms of intracrinology. Diethylstilbestrol is a synthetic nonsteroidal estrogen used in the treatment of menopausal and postmenopausal disorders. These compounds are typical examples of the myriad steroids that may be found in environmental systems.

For the extraction of steroids from water, stir bar sorptive extraction (SBSE), liquid-liquid extraction (LLE) and solid phase extraction (SPE) are typical of the techniques that have been successfully employed[24,25]. However they suffer from high labor intensity, lack of simple automation, high solvent and consumable use and the general need for internal standard quantitation.

Solid-phase micro-extraction (SPME) has been used in numerous fields such as: food analysis, including juices, soft drinks, dairy beverages, alcohols fruits and vegetables for volatiles, aroma, caffeine, fatty esters[26], environmental applications, including volatile organic

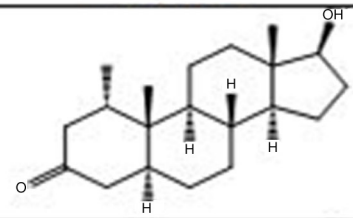
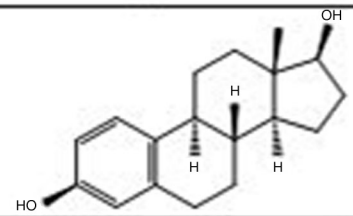
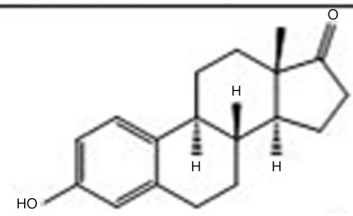
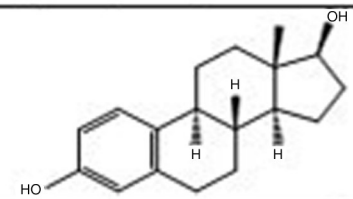
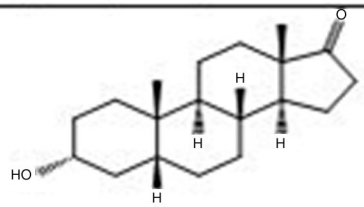
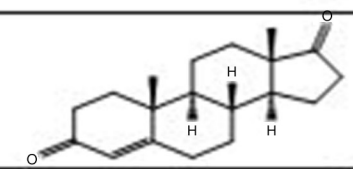
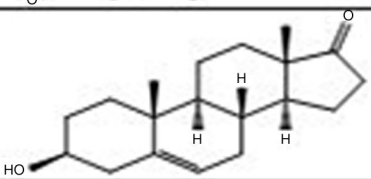
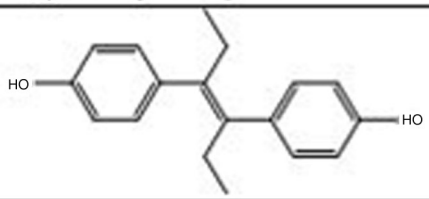
Steroids	Structure
Mesterolone	
Methandriol	
Estrone	
Estradiol	
Eticholan-3α-17-one	
Androsten-3-ene-17-dione	
Prasterone	
Diethylstilbestrol	

Figure 1. Structures of steroids used in this study.

contaminants, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, phenols, pesticides, steroids, herbicides, acid/neutral/base compounds, and organometallic compounds from water, air, soil, sludge, and sediment samples[27]. In drug analysis, SPME has been used for extraction of amphetamine, cannabinoids, cocaine, opiates, anesthetics, antibiotics, antidepressants and others from urine, blood plasma and hair[28]. These few examples provide a sampling of the large variety of SPME analyses performed over the past 20 years.

In recent work, SPME of steroids has involved interfacing to more sophisticated instrumentation and non-traditional adsorbent materials and geometries. Qiu et al. developed a selective SPME fiber for anabolic steroids from a testosterone molecular imprinted polymer (MIP)[29]. The fibers were used for the extraction of anabolic steroids and then analysed with GC–MS. LODs of 0.023 and 0.076 mg L^{-1} were obtained by Jiang et al. for the determination of estrogenic compounds (17α-estradiol, estriol, and diethylstilbestrol) in fish and prawn tissue by using an MIP-coated SPME fiber coupled directly to LC-UV for simultaneous multi-residue monitoring of the estrogens[30]. Wen et al. developed an on-line method for the simultaneous determination of four endocrine disruptors (17α-estradiol, estriol, bisphenol A and 17α-ethinylestradiol) in environmental waters by coupling in-tube SPME to LC with fluorescence detection[31]. Recent applications in in-tube SPME include the use of monolithic capillaries for the analysis of illicit drugs in urine and plasma samples[32].

In DI-SPME, for extraction of semi- and non-volatile analytes from water, a two phase equilibrium exists for the analyte between the liquid phase and the SPME fiber. The fiber/sample partition coefficient and apparent partition coefficient represent the quantitative amount of the analyte extracted and are direct indicators of the extraction efficiency of the fiber. The fiber/sample extraction ratio can be determined by knowing the concentration of the analyte sorbed by the fiber to the concentration of analyte remaining in the sample matrix after extraction. The conventional methods used for the determination of extraction ratio and partition coefficient require the initial concentration of the analyte and a liquid injection of sample for calibration[33,34]. The method published by Zimmerman, et al employed depletion study to determine the fiber/sample partition coefficient for HS-SPME[35]. This method was adapted to determine the fiber/sample apparent partition coefficient.

The high sensitivity and selectivity generated by MS-MS provides an ability to detect analytes at trace levels. A comparison study between triple quadrupole (QqQ), time of flight and hybrid quadrupole time of flight mass analyzers coupled to liquid chromatography was done for the detection of anabolic steroids in doping control analysis[36]. In this study, QqQ allowed the detection of all analytes at the minimum required performance limit. Santena et al. validated a gas chromatography/tandem mass spectrometry assay (GC-MS-MS) for estradiol and determined estrogen levels in normal post-menopausal women and in women with breast cancer before and during administration of aromatase inhibitors. Levels of estradiol approximately 10 pg/mL were detected[37].

Generally, low-level steroids analysis is done by LC- tandem mass spectrometry with solid phase extraction. As seen above, SPME-GC-MS-MS has not been applied to low-level steroids analysis; in this work, we demonstrate the effectiveness and potential for SPME-GC-MS-MS for ultra-trace analysis of steroids at similar concentrations to those determined by LC-MS-MS and related techniques.

2. Experimental

2.1. Materials and Chemicals

Ultra-pure water was obtained from a MilliQ Plus purifier (Millipore, Billerica, MA). The steroids used for this work were purchased from Sigma-Aldrich (St. Louis, MO): 1 alpha-methyl-17 beta-hydroxy-5 alpha-androstan-3-one (Mesterolone), 3S,8S,9R,10R,13S,14R,17S)-10,13,17-trimethyl-1,2,3,4,7,8,9,11,12,14,15,16-dodecahydrocyclopenta[a]phenanthrene-3,17-diol

(Methandriol), (8R,9S,13S,14S)-3-hydroxy-13-methyl-6,7,8,9,11,12,13,14,15,16- decahydrocyclopenta[a] phenanthren- 17- one (Estrone), (17β)-estra-1,3,5(10)-triene-3,17-diol (Estradiol), 4-Androstene-3,17-dione (Androstendione), 3α-Hydroxy-5β-androstan-17-one (Etiocholan-3α-ol-17-one), (3S,8R,9S,10R,13S,14S)-3-hydroxy-10,13-dimethyl-1,2,3,4,7,8,9,11,12,14,15,16-dodecahydrocyclopenta[a]phenanthren-17-one (Dehydroepiandrosterone or Prasterone) and 4,4'-(3E)-hex-3-ene-3,4-diyldiphenol (Diethylstilbestrol). Sodium phosphate dibasic and potassium phosphate monobasic, sodium chloride and sodium hydroxide pellets were purchased form Merck (Darmstadt, Germany). A Maxi Mix II vortex mixer (Barnstead, Des Moines, IA) was used to vortex the vials.

A TRACE 1310 gas chromatograph with MS-MS (TSQ Quantum Ultra) and TriPlus RSH autosampler with SPME capability was provided by Thermo Scientific (Dallas, TX) for this work. Polydimethyl siloxane-divinyl benzene (PDMS-DVB) SPME fibers were obtained from Sigma Aldrich (Supelco, Belfonte, PA). 20 ml vials with screw caps were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Buffer, Standard and Sample Preparation

Phosphate buffer (0.5 M) was prepared in water by dissolving 3.6 g sodium phosphate dibasic and 3.0 g potassium phosphate monobasic in deionized water to make 1.0 L of solution and adjusted to pH 8.0 using 5M sodium hydroxide. Stock solutions of each steroid with concentration of 100 µg/ml were prepared in ethanol. From the stock solution 10 µg/ml of steroids mix was prepared and was refrigerated in amber colored vials. The stock solutions were used within 15 days of preparation. For standards, 4.3 g of salt, 17 ml of water and 50 µL of phosphate buffer were added to a 20 mL vial. The final analyte concentration was attained by spiking a calculated volume of the stock solution into the vial. Each vial was then vortexed until the salt was dissolved completely. For real samples, the same procedure was used except without spiking with steroids.

2.3. Instrumental Conditions

2.3.1. SPME Conditions

The optimized SPME conditions were chosen from previous work done by Gomes et al. in our laboratory[38]. A PDMS-DVB fiber (65 µm) was used as sorbent in direct immersion-SPME mode. The vial was pre-incubated in the agitator for 10 min at 55°C, and then was extracted in sample vial for 60 min followed by desorption in the GC inlet under splitless conditions for 3 min. Prior to each analysis, the fiber was pre baked for 25 min and following each analysis, post-baked for 16 min at 260°C in the SPME fiber conditioning station on the autosampler.

2.3.2. GC-MS-MS Conditions

The inlet was maintained at 250°C in splitless mode with purge time of 3.00 min and a 0.75 mm inside diameter glass sleeve. The column was RTX-5MS, 15 m × 0.25 mm × 0.25µm (Restek, Bellefonte, PA). A constant flow of 1.5 ml/min was maintained throughout the analysis. The oven temperature program started at 40°C with hold time of 1 min and programmed at 20°C/min to 300°C with final hold time of 3 min. The total run time was 17.00 min. The transfer line was maintained at 250°C and the ion source was kept at 250°C. The steroid mixture was first analyzed in full scan, followed by product ion scans of each analyte to optimize the collision energy, collision gas pressure and identify the quantitation and conformation ions, as shown in Table 1. The initial method conditions were optimized at the 5 ppb concentration level. The final optimized experiments were performed selected reaction monitoring mode (SRM).

2.4. Depletion Study

A 0.1 ng/mL solution of the steroid mixture was prepared as mentioned previously in the sample preparation procedure. DI-SPME was performed consecutively for six times using the same solution containing the steroid mixture and desorbed in the inlet after every extraction. A plot of log peak area against

extraction number was generated. The resulting plot was examined for linear behavior and analysis to determine the partition coefficient according to the method of Zimmerman et al.[35].

2.5. Validation

The developed method was validated as per IUPAC guidelines[8]. Precision of the method was calculated by using % relative standard deviation of 5 runs. LOD was determined using the IUPAC method, using the equation shown below

$$LOD = ks_B / m \quad (1)$$

where k the S/N threshold which is equal to 3 for the LOD and equal to 10 for the LOQ. *sB* is the standard deviation of the blank which was determined taking the standard deviation of the noise readings from 10 data points adjacent to the peak at S/N between 2 and 3. m is the slope of calibration curve. The percent recovery from each sample was determined by first analyzing a 100 pg/mL sample and using that response and the calibration curve to estimate the expected response of a 600 pg/mL sample. This was then compared to the response of an actual 600 pg/mL sample obtained by spiking the original 100 pg/mL sample the ratio expressed as a percentage. Five real samples were obtained from various locations in the environs of South Orange, NJ USA.

3. Results and Discussion

3.1. Solid Phase Microextraction (SPME)

As steroids are semi volatile compounds with various polar functional groups, direct immersion solid phase micrextraction was performed with a commercially available semi polar fiber, 65 µm polydimethylsiloxane/ divinylbenzene (PDMS/DVB). The SPME procedure used was adapted from Gomes et al.[38]. A brief summary of parameters is presented here.

4.3 g of sodium chloride was added to the samples to take advantage of the "salting out" effect, increasing the amount extracted by reducing solubility of the analytes in water. As the steroids used all had pKa >10, samples were prepared using a phosphate buffer with pH = 8 to ensure that the steroids were predominantly in the neutral form to enhance extraction. Extraction time and temperature were optimized at 60 min and 55°C respectively, providing faster kinetics through the higher temperature without excessive reduction in the amount of analyte extracted, as partitioning into the fiber is usually favored by lower temperatures.

3.2. GC-MS-MS

RTX-5MS, 95% polydimethyl siloxane and 5% diphenyl polysiloxane, a very common stationary phase, was used for the chromatographic separation. This column was used as the first dimension column in the GCxGC-ToFMS work of Gomes et al.[38]. In that work, the second dimension column was used to separate the same co-eluting analytes. In this work, co-eluting analytes are quantified separately using the multiple reaction monitoring capability of the MS-MS detector. Figure 2, showing the co elution of several steroids, is a full scan chromatogram of the analysis of a 5 ppb steroid mixture. The steroids are identified in the figure caption and in Figure 1. Note the similarity in the steroids' structures and the low selectivity of the chromatographic separation. Co-eluting analytes may either be further separated chromatographically (changing parameters in GC or GCxGC) or by simultaneous selective detection (GC-MS or GC-MS-MS).

To determine the appropriate ions for the product scan in MS-MS the full scan mass spectrum and retention time for each steroid were determined. From the mass spectra, the most stable ion for each steroid was chosen (usually the molecular ion). The product scan in Q_3 was performed on the steroid mixture at varying collision energy and collision pressure to reduce the most abundant m/z value to 10% of its original height according to the manufacturer's recommendation to increase abundance of product ions. The two most stable transitions were chosen as the confirmation and quantitation ions. The

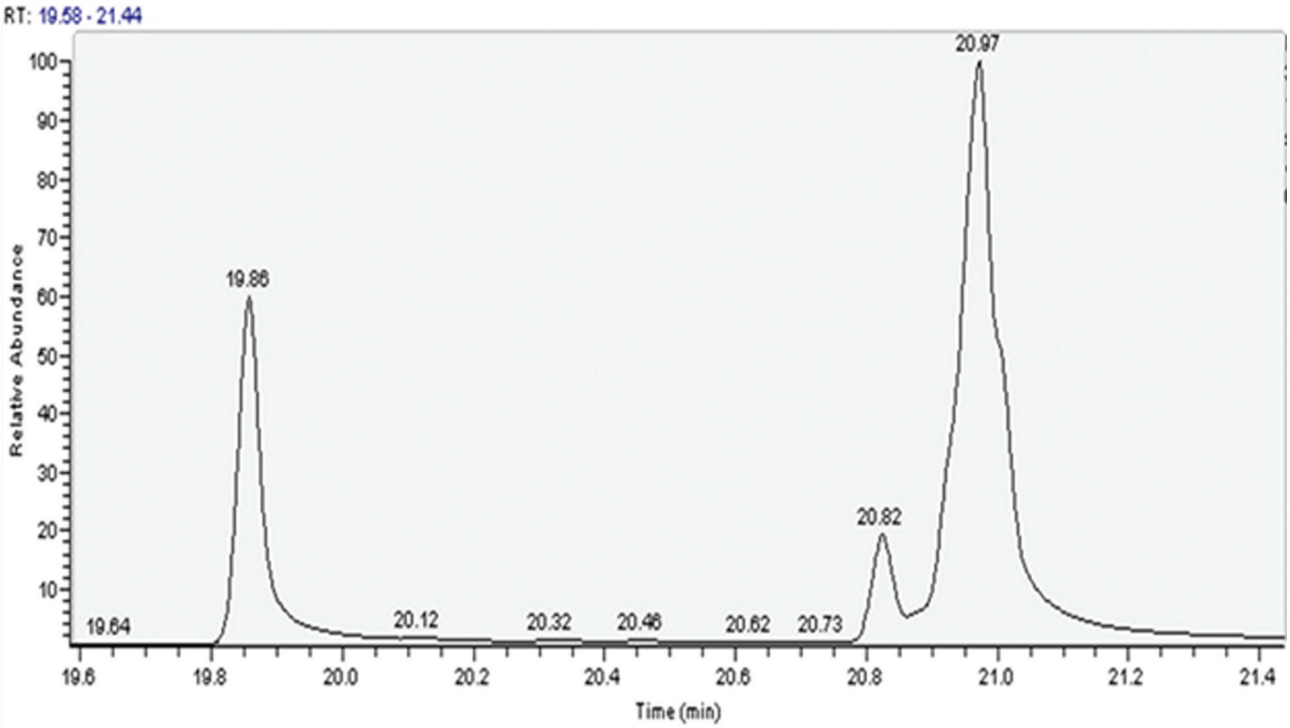


Figure 2. Total ion chromatogram of a 1 ng/mL steroid mixture, extracted according to this work, except 10°C/min temperature ramp. Peaks are not identified due to the severe peak overlap. See Figure 3 for the identities of the separated components.

optimized conditions obtained from the product scan were used in selected reaction monitoring, as shown in Table 1. The next step was to apply those optimized conditions to selected reaction monitoring (SRM), with both quadrupoles Q1 and Q3 set at specific m/z for each steroid, maximizing selectivity of the mass analyzer.

Table 1. Optimized MS-MS Conditions; Parent Ion, Product Ions (quantitation ion listed above confirmation ion), Collision energy (eV) and Chromatographic Retention Time (min).

Steroid	Parent	Product*	Collision Energy	Retention Time
Mesterolone	218	159 185	12	13.18
Methandriol	253	183 197	20	12.89
Estrone	270	172 185	16	13.05
Estradiol	272	186 213	15	13.11
Androstendione	286	109 124	12	13.16
Eticholan-3-17-one	290	244 257	10	12.56
DES	312	203 270	10	12.69
Prasterone	288	240 269	14	12.75

*Quantitation ion is listed above product ion.

Figure 3 shows ion chromatograms for each of the steroid compounds at the 1 ppb concentration level, showing well separated and resolved mass peaks for each steroid. Each peak was fully separated and demonstrates excellent chromatographic performance, symmetrical peak shape and narrow peak width and the mass chromatogram of each steroid is "clean" with very low noise, high signal and no interferences. By using selected reaction monitoring, the co-elution problem was solved through multidimensional mass detection. SRM allowed selective monitoring of the specific transitions for each steroid while maintaining sensitivity during operation at less than unit mass resolution. The quantitative results of this approach are demonstrated later in this work in the validation section.

3.3. Fiber/sample partition coefficient by depletion study

Successive extractions from the same vial at an initial 100 pg/mL concentration were performed. A plot of log peak area versus extraction number showed a linear response with a correlation coefficient greater than 0.98 for most of the steroids. The fiber/sample partition coefficient was then determined using the method of Zimmerman, et al.[35]. The extraction ratio was calculated from the slope of the linear regression and the apparent fiber/ sample partition coefficients for the steroids were calculated from the extraction ratio. The extraction ratio and the fiber/sample partition coefficient are presented in Table 2. The partition coefficients were all greater than 10,000, supporting the high sensitivity of this technique.

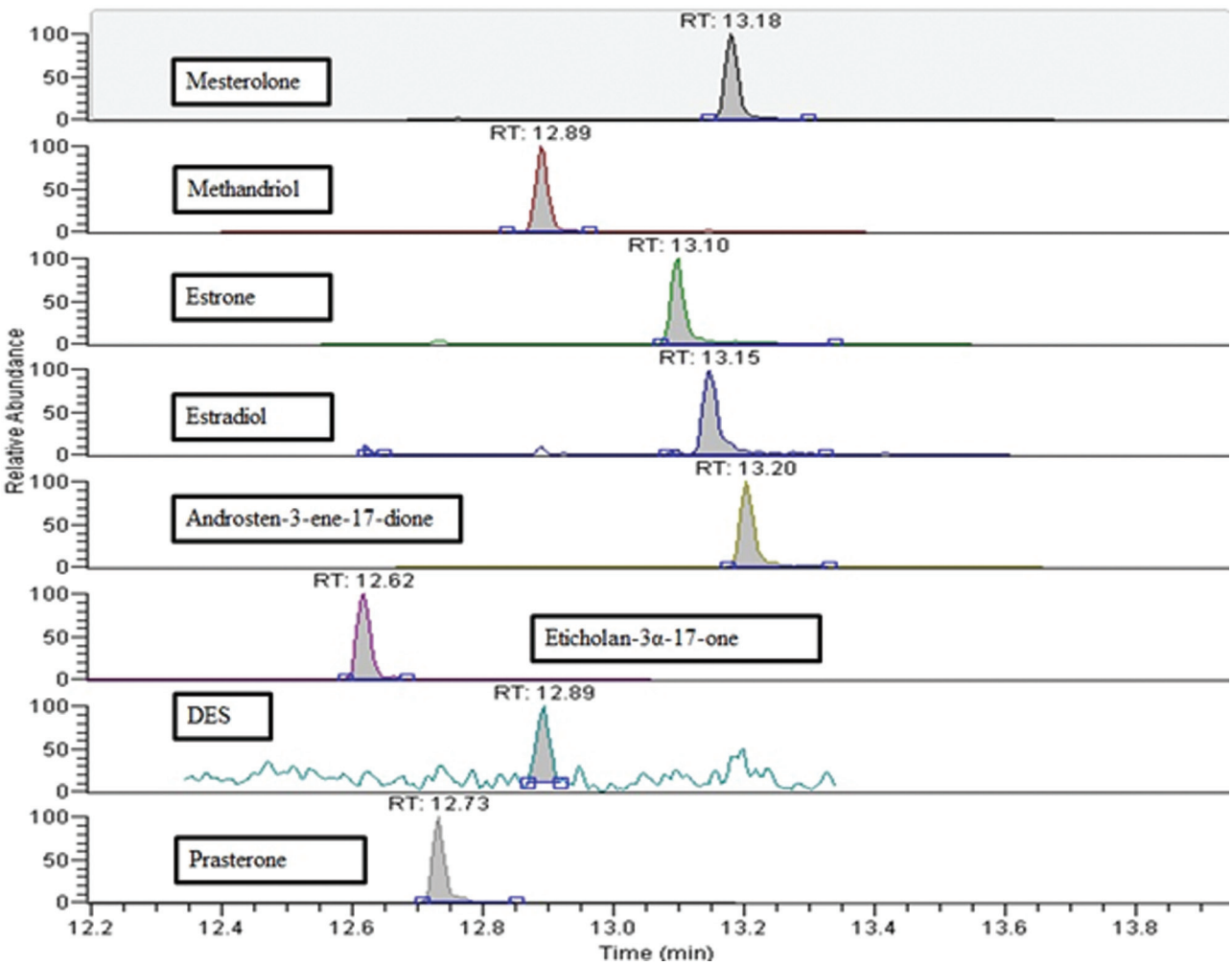


Figure 3. Ion chromatograms of the steroid mixture extracted at the 1 ng/mL level.

Table 2. Extraction ratio and fiber/sample apparent partition coefficient at 55°C. Values calculated according to the method of Zimmerman, reference 35.

Steroid	Correlation Coefficient	Slope	Extraction ratio	Fiber /Sample apparent partition coefficient (x 10^4)
Mesterolone	-0.9924	-0.145	0.28	1.5
Methandriol	-0.9909	-0.132	0.26	1.4
Estrone	-0.9859	-0.116	0.23	1.2
Estradiol	-0.9763	-0.105	0.21	1.1
Androstendione	-0.9942	-0.103	0.21	1.0
Eticholan-3-17-one	-0.9873	-0.145	0.28	1.5
DES	-0.9130	nc*	nc*	nc*
Prasterone	-0.2716	nc*	nc*	nc*

*nc : Not calculated.

Table 3. Analytical figures of merit and quantitative validation results.

Steroid	R^2	% RSD	Recovery (%)	LOQ (pg/L)	LOD (pg/L)
Mesterolone	0.9975	7.98	103	1200	200
Methandriol	0.9978	9.20	96	200	30
Estrone	0.998	7.80	85	2000	220
Estradiol	0.9955	9.92	85	1000	200
Eticholan-3α-17-one	0.9977	9.34	84.9	900	300
Androsten-3-ene-17-dione	0.9973	7.30	96.5	1000	200
Prasterone	0.9972	10.80	88	250	30
Diethylstilbestrol	0.9837	9.42	92	5000	2000

DES did not show a linear curve. This is because the signal to noise ratio for DES was lower, even during the first extraction. For this reason the partition coefficient was not calculated. As PDMS-DVB operates on an adsorption mechanism, competition for adsorption sites may explain the loss of response for DES. Mesterolone and Eticholane-3α,17-one showed the highest fiber/sample apparent partition coefficient at 55 °C under the given extraction conditions. The apparent partition coefficients for all of the steroids were above 10,000 demonstrating high affinity of the PDMS DVB fiber for steroid extraction.

3.4. Validation

Table 3 shows analytical figures of merit for all the steroids. Traditionally, SPME is performed using internal standard quantitation, to account for run-to-run variation in extraction and injection. However in this work, the precision data were less than 10% RSD at the 1 ng/mL level for all steroids, so further analysis was performed without using an internal standard. Calibration curves were plotted for each steroid using external standard calibration. Additionally, steroids are often derivatized prior to analysis by GC-MS to improve extraction, chromatographic or spectrometric performance. The use of SPME eliminated the need for derivatization, eliminating the potentially adverse effect on reproducibility that is often introduced by derivatization. Calibration was plotted over a wide range of 0.01 ng/ml to 5 ng/ml. All steroids showed $R^2 > 0.99$ and % RSD less than 10% at 1 ng/mL. The LOD and LOQ's in pg/L illustrate the potential of SRM at ultra-

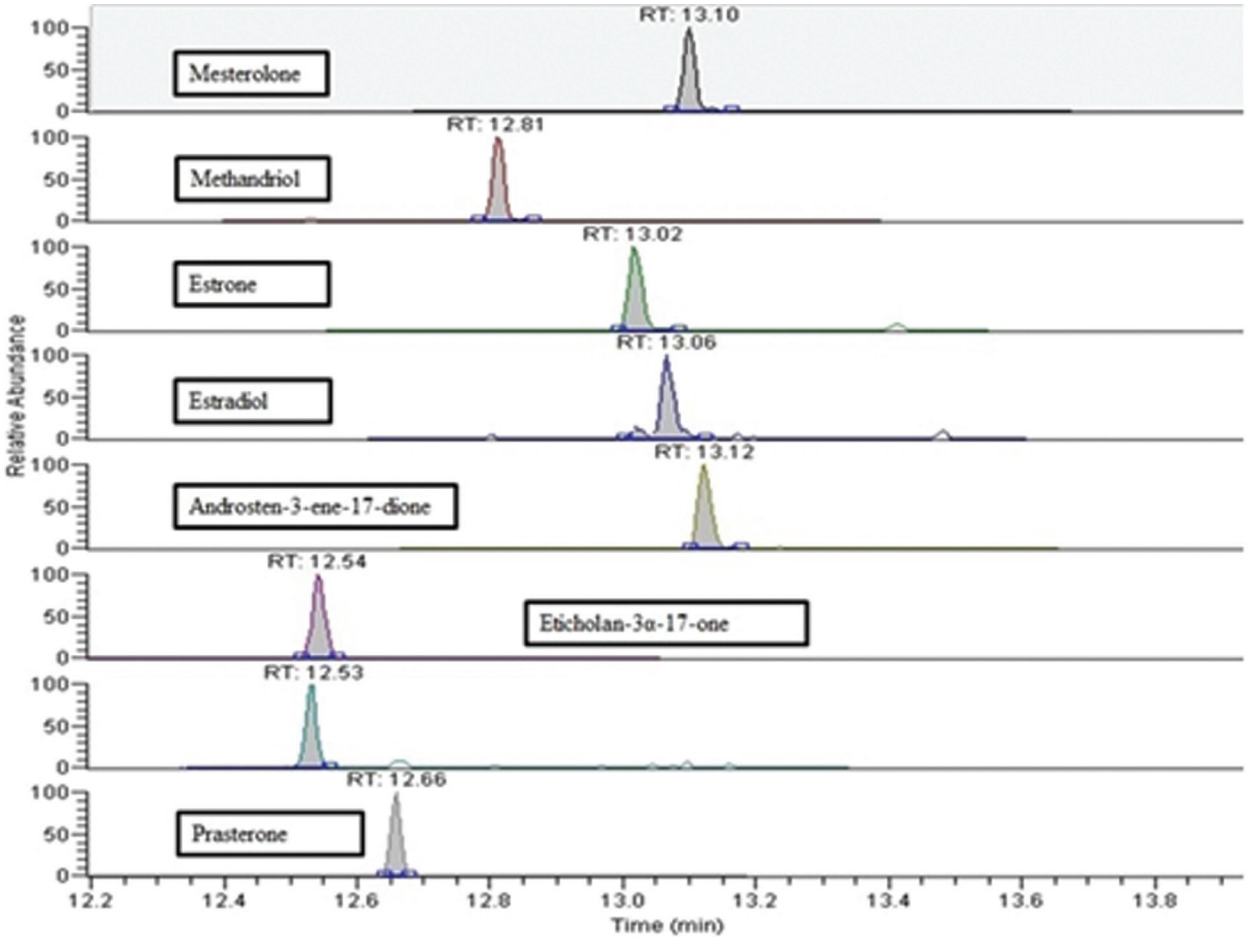


Figure 4. Ion chromatograms of the extracted steroids at the 3 pg/mL level.

trace levels. As an example, Figure 4 shows mass chromatograms for the steroids at the 3 pg/mL level, still showing strong signal to noise rations, easily indicating detection limits in the sub-pg/mL range.

Five water samples were collected from local sources near South Orange, NJ USA. One sample showed a small response (pg/L level, with S/N about 10) for estrone. All of the other samples were fully negative for all of the analytes. This being a highly selective method, there is no way to determine whether other steroids or compounds that may be of interest were present from these data. However, this does demonstrate the potential for SPME-GC-MS-MS as an effective technique for the low level analysis of drugs in environmental water samples.

4. Conclusions

SPME- GC-MS-MS in selected reaction monitoring mode was used for the trace analysis of steroids in water. The method was calibrated from 0.01 ng/ml to 5 ng/ml, with limits of detection and quantitation below 1 pg/mL for all of the steroids determined. The method showed good chromatographic figures of merit and quantitation at pg/ml concentration without the use of any internal standard or derivatization. This is attributed to effective extraction using automated SPME which ensures precision by eliminating analysis variables in sample preparation and MS-MS which allows monitoring of transitions specific to each steroid, delivering high selectivity and resulting low noise. Monitoring compounds at less than unit mass resolution values provides the

selectivity necessary for analyzing multiple overlapping compounds without compromising the sensitivity needed to determine analytes at pg/mL and lower concentration. Additionally, the fiber /sample apparent partition coefficient was determined for six steroids at 55°C, using a depletion study to be greater than 10,000, supporting the ability of SPME to extract detectable amounts of the analytes from very low concentration samples.

Acknowledgments

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SPME-GC-MS determination of selected aniline pesticides in surface water

Carin von Mühlen[1,2]
Fernando Mauro Lanças[1*]

[1]University of São Paulo – USP,
Institute of Chemistry at São Carlos
13560-970 São Carlos (SP) Brasil
[2]Present address: Universidade Feevale,
Instituto de Ciências Exatas e
Tecnológicas, RS-239, 2755 - Vila Nova
93352-000 Novo Hamburgo (RS) Brasil
*flancas@iqsc.sc.usp.br

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Abstract

An analytical methodology to determine Alachlor, Butralin, and Metolachlor herbicides, and Metalaxyl fungicide, in surface water was developed using Solid Phase Microextraction (SPME) and Gas Chromatography coupled to Mass Spectrometry (GC-MS). The developed method was validated, and the quantification limits were found to be between 0.2 to 1.8 $\mu g.L^{-1}$, depending upon the compound. The precision, as measured by the relative standard deviations (RSD), was in the range 3.5% to 27%. The higher RSD was found for Butralin due to the occurrence of adsorption of this compound in the extraction fiber. After validation the developed methodology was employed to the determination of these four pesticides in water samples that supplies Araraquara and São Carlos cities (São Paulo - Brazil). Samples from Feijão Creek, Anhumas Creek, Cruzes Creek and Paiol Brook, as well as samples from after-treatment at SAAE (Water and Sewer Supply Service – São Carlos, SP, Brazil) and DAAE (Water and Sewer Supply Department – Araraquara, SP, Brazil) were evaluated. Samples from both winter and summer seasons were collected and analysed. The results show that the samples analyzed using the in-house validated methodology were not contaminated with these pesticides within the investigated concentrations.

Keywords: water, anilines, pesticides, SPME, GC-MS, herbicides.

"Dedicated to Professor Harold McNair on the occasion of his 80th birthday".

1. Introduction

Water is extremely important to the Earth ecosystem. The relevance of water quality preservation is constantly increasing. The intensification of agricultural production, the monoculture cultivation to the human nourishment, and the industrial process have been requiring a proper regulation of pesticide uses.

The Economic Commission for Europe established 0.1 $\mu g.L^{-1}$ as the maximum admissible concentration of any pesticide in human consume destination water and 0.5 $g.L^{-1}$ to the total residues. However, is not clear if the transformation products are also included in this directive[1]. This limit have been polemic because it did not consider the toxicity of each product, and for some compounds, the available analytical methodologies do not achieves detection limits in this order. On the other hand, the USA Environmental Protection Agency (US-EPA) and the Health World Organization (HWO) established maximal levels to individual pesticides in human consume destination water, based on toxicological and epidemiological studies. EPA methods used to determine Alachlor (methods 505, 525.1, 525.2, 507 and 551.1) and Metolachlor (Methods 507 and 551.1) in water, among other compounds, are described in the literature[2,3] but robust, quick and cheap methods to determine Butralin and Metalaxyl were not found.

In Brazil, CONAMA Resolution 357[4], established contaminants maximal limits in water to various destinations, which include the evaluation of some Organochlorine, Organophosphorous and Carbamates pesticides. Resolution 36/GM from the Health Ministry established limits for pesticides in human consume destination water. However, the majority of pesticides nowadays used, such as Pyrethroids insecticides and several herbicides, are not included in these regulation.

Since beginnings of 70´s, the Brazilian agriculture, in particular the agriculture performed in São Paulo state, have been suffering major technological changes, including the replacement of workers by machines and, especially, a large increase in the use of chemical products. At that time, Rodrigues and coworkers[5] related that the use of pesticides was higher than 78000 ton/year of applied active principle, or 2.5 kg/ha harvest.

Sanchezbrunete and coworkers[6], gives one account that the control of noxious weeds in corn plantation is done using herbicides such as Atrazine and Chloroatrazine in binary mixtures with Chloroacetamide herbicides as Alachlor and Metolachlor. The environmental contamination power of theses herbicides comes from their physical-chemistry properties and the culturally used practices. Higher concentrations of theses herbicides generally are detected in surface and underground water, in different countries (Economic Commission for Europe, 1992).

Cholotriazin, Choloroacetamides and Nitroanilines analysis usually have been done by gas chromatography (GC) with nitrogen-phosphorous detector (NPD) or electron-capture detector (ECD) and, more recently, by reversed phase liquid chromatography[7-10]. Residues confirmation have been done, in most cases, by GC-MS and less frequently by LC-MS(MS). Solid Phase Microextraction (SPME) has been used for pesticide determination in water[11-14] coupled with both GC or LC. SPME is a fast and simple technique that minimizes sample preparation steps, reducing the analysis time consuming, systematic errors and sample contaminations, as earlier shown by Pawlyszyn and coworkers[15].

In this work, an analytical methodology to determine Alachlor, Butralin, and Metolachlor herbicides, and Metalaxyl fungicide (Figure 1) in surface water has been developed, validated and applied to the analysis of these pesticides in water samples.

Metolachlor, *2-ethyl-6-methylaniline* is an organic compound widely used as a herbicide. It is a derivative of aniline and is a member of the chloroacetanilide herbicides. It has been detected in ground and surface waters and various concentrations throughout the U.S. It is classified as a Category C pesticide by the United States Environmental Protection

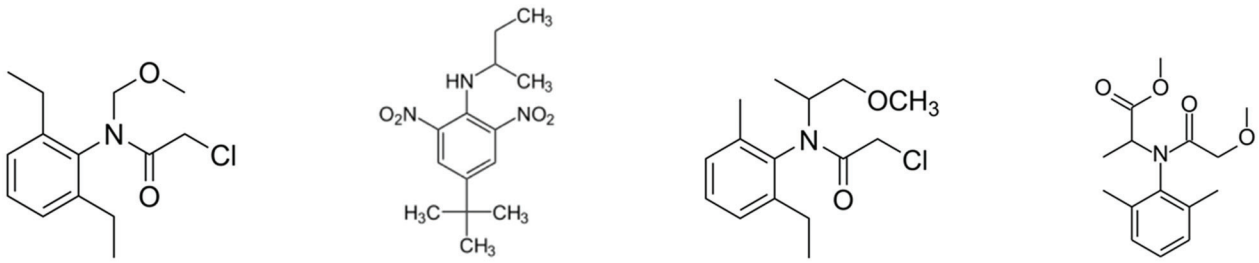

Figure 1. Chemical structure of the selected pesticides evaluated in this study.

Agency (US EPA) which indicates limited evidence of carcinogenicity. *Alachlor, 2-chloro-N-(2,6-diethylphenyl)-N-(methoxymethyl) acetamide*, is a herbicide used for weed control on corn, soybeans, sorghum, peanuts, and beans. It has been evaluated for carcinogenic activity in rats and mice. In accordance with the EPA proposed Guidelines for Carcinogen Risk Assessment, Alachlor was classified as "likely" to be a human carcinogen at high doses, but "not likely" at low doses. *Metalaxyl, methyl N-(methoxyacetyl)-N-(2,6-xylyl)-DL-alaninate*, is a phenylamide fungicide with systemic function, used to control Pythium in a number of vegetable crops, and Phytophthora in peas among other applications. The oral and dermal LD50 in rats indicates slight toxicity by ingestion and dermal application. No information was available regarding the inhalation toxicity of metalaxyl. *Butralin, (RS)-N-sec-butyl-2-6-dinitroaniline*, is a weak oxidizer herbicide, stable to hydrolysis and somewhat to photolysis. It is very toxic in contact with skin, causing sensitization by skin contact, and irritating to eyes, respiratory system and skin. It is considered very toxic to aquatic organisms may cause long-term adverse effects in the aquatic environment. As a consequence of the toxic characteristics to humans and the environment presented by these compounds, and the large use of pesticides in Brazil at the presente time, their analysis in water samples is imperative.

The methodology in this work, based on SPME as the extracting, clean-up and concentration technique, followed by GC-MS, was validated and the analytical determination of these four pesticides in water samples that supplies Araraquara and São Carlos cities (São Paulo - Brazil) from both winter and summer seasons, was done. Samples from after-treatment at SAAE (Water and Sewer Supply Service – São Carlos, Brazil) and DAAE (Water and Sewer Supply Department – Araraquara, Brazil), from both winter and summer seasons, were also collected and analysed.

2. Materials and Methods

Pesticide analytical standards were purchased from Supelco (Bellefonte, PA, USA), with purity grade higher than 97.1%. To prepare the standard solutions acetone P.A. from Carlo Erba (Milano, Italy) and Milli-Q grade water from Millipore (Bedford, MA, USA) were used. In order to study the ionic strength effect on the extraction, sodium chloride P.A. from Merck (Apartado, Darmstadt, Germany) was used. For the SPME experiments, a holder and poliacrilate fibers (85 μm film thickness), both purchased from Supelco were used. Stir bars and 5 mL conical botton glasses both from Kimax (Kansas City, MO, USA), and a magnetic stirring Corning (São Paulo, SP, Brazil), as well as a thermostatic bath Quimis (Diadema, São Paulo, Brazil). The glass materials were silanized according to Doong and coworkers[16] procedure, using methanol, acetone, hexane and toluene from Mallinckrodt (Phillipsburg, NJ, USA), a 10% (v/v) mixture of dichlorodimethylsilane (Supelco) in toluene and an oven from Fanem (São Paulo, SP, Brazil). Analyses were performed on a

Shimadzu QP5050A (Shimadzu, Kyoto, Japan) gas chromatograph-mass spectrometry system. Pesticides were separated on a DB5 column from J & W Scentific (Agilent, São Paulo, Brazil) having the following dimensions: 30 m × 0.25 mm I.D. × 0.30 µm film. The split-splitless injector was operated in the splitless mode. The injector port and detector temperatures were 523 K and 553 K, respectively; Helium was used as carrier gas at 30 cm/sec. The column temperature was held at 343 K for 2 min, increased to 493 K at rate of 10 $K.min^{-1}$, increased to 543 K at rate of 2 $K.min^{-1}$, increased to 553 K at rate of 5 $K.min^{-1}$, with the final temperature held for 4 min. The detector was operated in the Single Ion Monitoring (SIM) mode, with a detector energy of 1.5 eV and electronic impact of 70 eV. The monitored ions were of m/z 132, 160, 162, 188, 206, 238 and 266.

To develop the methodology a 100 mg/L stock solution of the four pesticides was used. The optimized parameters were extraction time of the compounds to the fiber, dessorption time on the GC injector, temperature extraction and salting out effect. Direct extractions were done, and the position of the fiber in the solution, stirring rate and position of the fiber on the injector were all maintained constant.

3. Results and Discussion

3.1. Method development

To determine the ideal extraction time, the temperature of extraction was fixed on 303 K and the fiber was maintained on the injector port during the whole chromatographic run. The immersion time should be sufficient to allow the fiber to absorb a significant quantify of analyte. The pesticide Butralin did not reach the equilibrium until 60 min, as shown in Figure 2, being the only compound evaluated that did not reach the equilibrium until 25 min. As Butralin presents a good detector response, a good compromisse between the extraction time and area counts was obtained after 20 minutes of exposure time. The minimum analyte carryover was obtained with 5 min of desorption time.

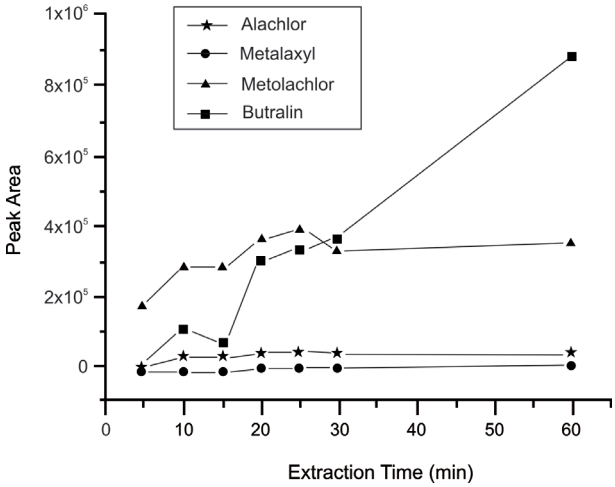

Figure 2. Variation of the area of the extracted analyte as a function of the extraction time using SPME.

As shown in Figure 3, the best temperature extraction for Alachlor, Metalaxyl and Metolachor was 303 K. Butralin presets a different performance again, being 303 K selected as the best extraction temperature for all compounds.

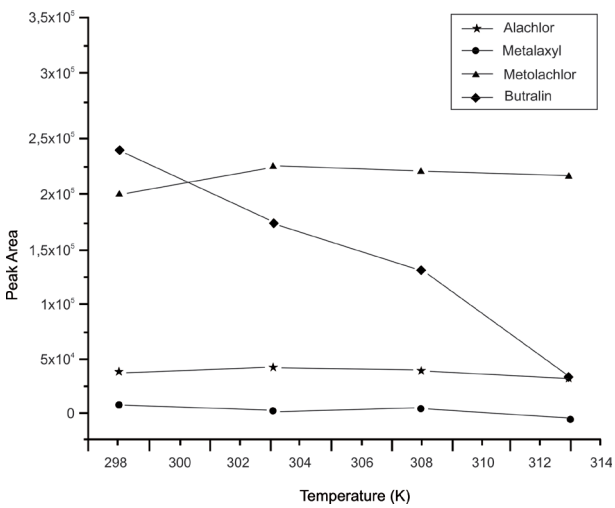

Figure 3. Effect of the temperature extraction on the pesticides recovery.

In order to study the salting out effect, NaCl was added to the standard solutions in concentrations of 20, 35, 60 $mg.mL^{-1}$, and saturated. As shown in Figure 4, Butralin presented a divergent behavior compared to the other compounds when salt was added. This

effect occurs probably because the two nitro groups could present a week ionic interaction that increases the matrix affinity when the ionic concentration in the solution increase. However, when the ionic concentration increase, the solvating power decrease, and the interaction between fiber and the other tree compounds increase. So, the average better salt concentration found was 60 $mg.mL^{-1}$.

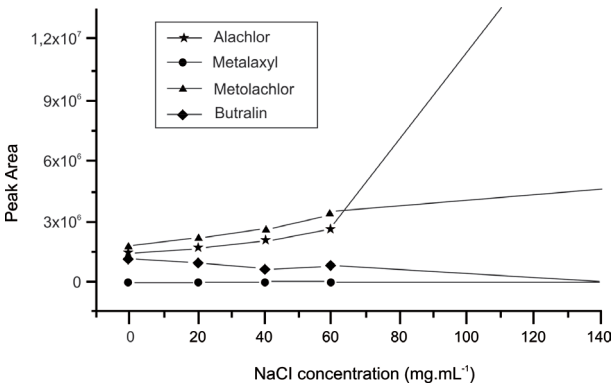

Figure 4. Salting out effect.

The final methodology assumed as the best condition amongst the ones investigated in this work has been as follow. Using 3 mL of sample and 180 mg of NaCl into a conical glass, the stir bar was inserted and the glass was closed with an aluminum paper film before the lid. This glass was inserted in the stirring and heating system, being under stirring (400 rpm) and constant temperature (303 K) for 10 min to achieve the equilibration. Then, the SPME system was inserted into the vial and the fiber was displayed inside the solution for 20 minutes, under constant stirring and temperature. The fiber was retracted from the solution and inserted in the GC injection port for 5 min after which the chromatographic run began.

3.2. Method Validation

To obtain the calibration curve, standard concentration of solutions containing the 4 pesticides in concentrations ranging from 0.1 $\mu g.L^{-1}$ to 100.0 $\mu g.L^{-1}$ were used. The methodology was applied tree times for each curve point, in order to generate an average value for each point. The straight lines equations obtained from calibration curve, the Linear Range (r), the precision (obtained with 12 analyses of 100$\mu g.L^{-1}$ solutions containing the 4 pesticides) as measured by the Relative Standard Deviation (RDS), the Detection Limit (LOD) and Quantitation Limits (LOQ) for each pesticide are shown on Table I.

As shown in Table 1, the Linear Range obtained for Metalaxyl is extremely poor, probably because this compound was retained in the stir bar, as confirmed in a separate experiment using blank (Milli-Q) water. Every glass material was silanized, but this compound did not disappear even in the blank extractions. The only one material that was not silanized at this step was the stir bar. When a blank experiment as done using the same apparatus, except that the stir bar was changed to a new bar, the Metalaxyl peak disappeared from the solution. A similar observation gave origen to a new extraction technique, named Stir Bar Sorptive Extraction (SBSE)[17], in which pesticides were observed to be retained in a stir bar coated using a polydimethylsiloxane film. To overcome this problem, which could also happen with other analytes, the use of silanyzed glass stir bar is higly recommended.

Table 1. Straight lines equations, Linear Range (r), Relative Standard Deviation (RDS), Detection Limit (LOD) and Quantitation Limits (LOQ) from each pesticide.

	Straight Line Equations	r	RSD (%)	LOD ($\mu g.L^{-1}$)	LOQ ($\mu g.L^{-1}$)
Alachlor	-693,8516 + 1302,95657 X	0.99752	3.86	0.00732	0.2196
Metalaxyl	9365,17892 + 19,85094 X	0.20391	15.66	0.4584	1.3753
Metolachlor	-1935,43355 + 2112,47939 X	0.99667	3.15	0.0942	0.2826
Butralin	-34206,10069 + 6691,86406 X	0.98751	27.22	0.6267	1.8801

3.3. Surface Water Analysis

Typical chromatograms obtained from the determination of theses pesticides in water samples that supplies Araraquara and São Carlos cities (São Paulo - Brazil) are shown on Figure 5.

The analysis were done with samples collected from Feijão Creek (I), Anhumas Creek (II), Cruzes Creek (III) and Paiol Brook (IV). Samples from after-treatment at SAAE (Water and Sewer Supply Service – São Carlos) (V) and DAAE (Water and Sewer Supply Department – Araraquara) (VI) are also analyzed. Samples from both winter and summer seasons were collected. A blank obtained with Mili-Q grade water (A), a crude sample (C), and spiked sample (D) containing a 10 $\mu g.L^{-1}$ pesticides solution, as well as a 10 $\mu g.L^{-1}$ standard solution containing the four pesticides studied (B) were analyzed for each sample collected. Each sample was analyzed in triplicate. As shown on Figure 5, in case they were present any one of these pesticides could be detected in the collected samples, using the developed methodology.

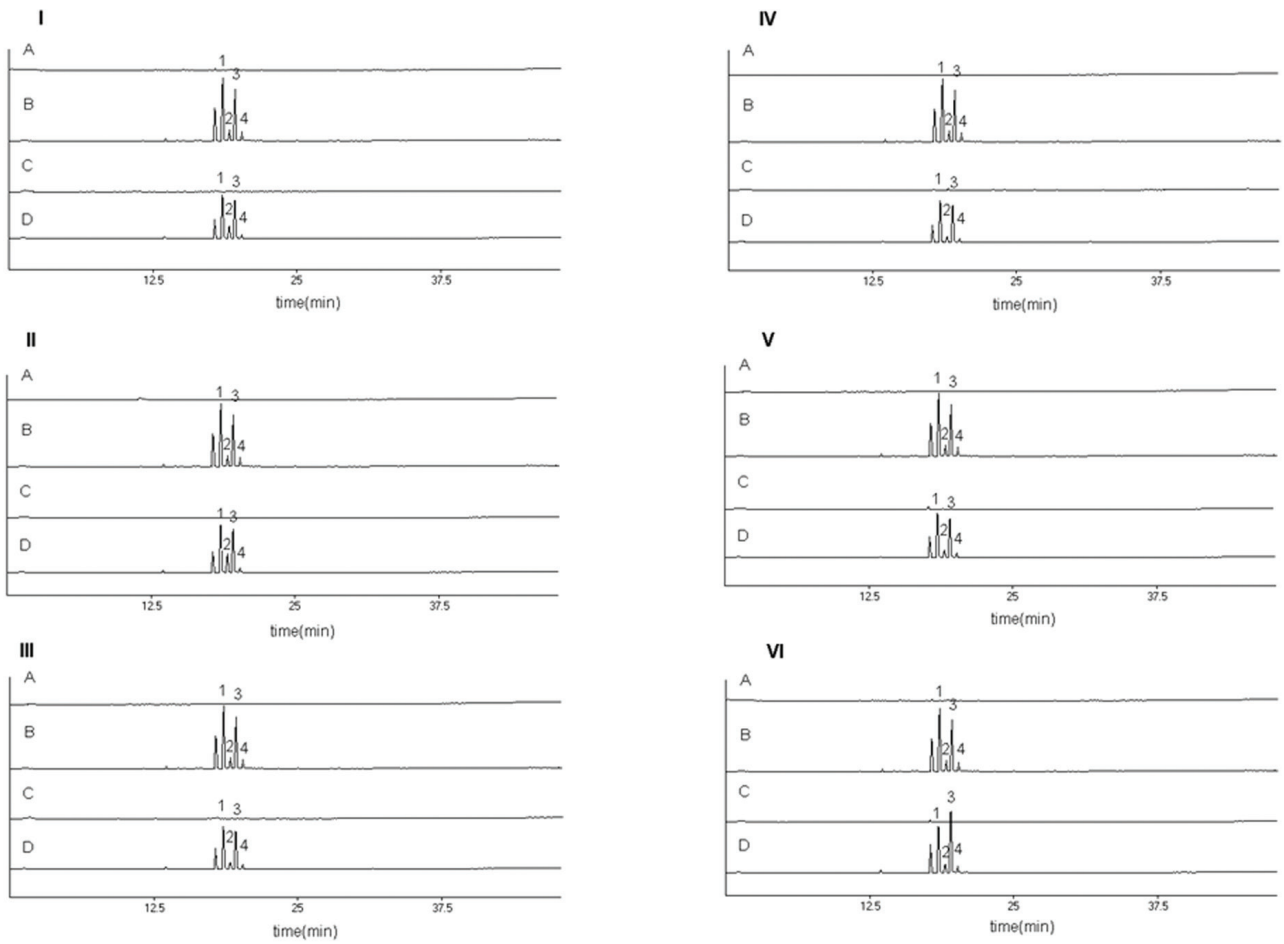

Figure 5. Chromatograms obtained for typical water samples. I - Feijão Creek; II - Anhumas Creek; III - Cruzes Creek; IV - Paiol Brook; V - after-treatment water sample from SAAE; and VI - after-treatment water sample from DAAE. A - blank (Milli-Q) water; B – (Milli-Q) water spiked with a 10 $\mu g.L^{-1}$ pesticides standard solution; C - water sample; D - water sample fortified with a 10 $\mu g.L^{-1}$ pesticides standard solution. Peaks: 1 = Alachlor; 2 = Metalaxyl; 3 = Metolachlor; 4 = Butralin.

4. Conclusion

The developed and validated methodology to determine Alachlor, Butralin, and Metolachlor herbicides, and Metalaxyl fungicide in surface water using SPME technology presented good precision for Alachlor and Metolachlor pesticides, and the detection limits obtained to these compounds are under the maximum limits established by the Economic Commission for Europe (0.1 $\mu g.L^{-1}$). Metalaxyl presented low precision due to its retention on the stir bar, as discussed. To solve this problem, silanized glass stir bar should be used. The presented results shows that the water samples both before and after-treatment at the Municipal Water Treatment Service at Araraquara and São Carlos cities (São Paulo - Brazil), sampled during both summer and winter, were not contaminated with these pesticides in the detectable concentrations achieved by the employed analytical methodology.

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SAMPLE PREPARATION

Comparison of Soxhlet extraction, ultrasonic bath and focused microwave extraction techniques for the simultaneous extraction of PAH´s and pesticides from sediment samples

Piotr Álvarez Porevsky
Humberto Gómez Ruiz*
Lucía Hernández Garciadiego

Facultad de Química,
Departamento de Química Analítica.
Universidad Nacional Autónoma de México.
México, D.F. 04510, México.
*email: hgomez@unam.mx

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Abstract

The need to develop an analytical procedure for the determination of pollutants in the Lacantun River at the "Montes Azules" Biosphere Reserve led to the evaluation of different extraction techniques. Because sediments are easier to preserve than water or biota, the evaluation of the quality of the water was assessed by analyzing the collected sediments. In order to preserve pristine rivers, water quality evaluation is fundamental. The Lacantun River borders the "Montes Azules" Biosphere Reserve, located in the state of Chiapas in Southwest Mexico. It is one of the few, perhaps the only, pristine river in Mexico. Due to the geographical location of the river, sampling and sample preservation result problematic. Sampling and analyzing the sediments in order to evaluate water quality was deemed the only solution for a sustained, monitoring of the river. In order to analyze the possible pollutants (polyaromatic hydrocarbons (16), organochlorine pesticides (11), organophosphorus pesticides (6) and permethrin) two extraction procedures where evaluated: Focused Microwave Extraction (FMWE) and Ultrasonic Bath Extraction (USBE). Both extraction techniques were compared with the classical Soxhlet Extraction (SE). In order to establish the optimum extraction conditions for USBE and FMWE techniques, a series of experiments guided by full factorial and fractional factorial designs were made. Both extraction techniques meet the validation criteria of determining a) coefficients above 0.7 in all cases and above 0.98 for 90% of the selected analytes; b) an RSD below 30% in all cases and c) a percentage of recovery between 70% and 130%. The microwave extraction and the ultrasonic extraction did not show a statistically significant difference for the extraction of PAH´s with MW<170g/mol; the Soxhlet extraction presented an efficiency 40% lower. For the rest of the PAH's (MW>170g/mol) there are no great differences between the extraction techniques. For the organochlorine pesticides the microwave extraction produced the best results, compared to the ultrasonic and the Soxhlet extraction which presented 8% and 20% lower efficiencies, respectively. The ultrasonic extraction gave the best results for the organophosphorus pesticides, whereas the microwave extraction and the Soxlet extraction showed efficiencies 8% and 40% lower. There was no significant statistical difference between the evaluated extraction techniques for permethrin. In the case of analytes extraction, the technique with the best efficiency was ultrasonic extraction. Additionally, it is also the most versatile technique due to its simple operation and fast extraction times when many samples are analyzed; also, it is cost-efficient and does not take up much laboratory space.

Keywords: sediments, focused microwave extraction FMWE, ultrasonic bath USBE extraction, soxhlet extraction, pesticides, polyaromatic hydrocarbons, GC/MS, multivariate optimization.

1. Introduction

The "Montes Azules" Biosphere Reserve in the state of Chiapas in Southwest Mexico is bordered by the Lacantún river. This river is one of the few –perhaps the only –river in Mexico, that has preserved its pristine conditions due to very low demographic concentration and the natural rainforest conditions of the area.

Currently, lands in the area surrounding the Reserve are under pressure to be used for farming and stockbreeding –a rather common phenomenon regarding Mexico's natural reservoirs.. These activities will deteriorate water quality, in order to preserve the site it is necessary to ascertain a pollution base line and monitor it periodically.

Due to the very low concentration of pollutants expected in the river water it is necessary to use systems that can concentrate persistent organic pollutants (POP´s), so that either the biota or the sediments remain. so that leaves either the biota or the sediments. The location of the Biosphere Reserve renders the sampling strategy critical. It is far away from the region's airports, which makes it very difficult to preserve biota samples cold all throughout transportation; because river sediments are better suited to withstand rough transportation conditions, water quality evaluation is done by means of sediment analysis.

Sediment analysis can be conveniently used to estimate point sources for pollutants that, upon being discharged into surface waters, do not remain in solution but are rapidly adsorbed by particulate matter, thereby escaping detection by water monitoring[1]. Due to their low aqueous solubility and highly hydrophobic nature, POP´s tend to associate with particulate matter and to accumulate in the sediments. Thus, river sediments that contain large pools of organic matter can constitute a significant repository for POPs[2,3].

Among the different methods available for the extraction of pollutants from sediments, microwave assisted extraction (MWAE) and ultrasonic bath (UB) techniques promised to provide the best recoveries -number of samples, water content, and type of pollutants expected where the main considerations –for the purposes of this project.

Both methods were evaluated using the classical Soxhlet extraction technique as a comparison standard.

2. Experimental

2.1. Reagents and chemicals

The solvents acetone and dichloromethane (pesticide grade), hexane (>95%, HPLC grade) and methanol (HPLC grade) were supplied by *J.T. Baker*. Magnesium silicate used for cleaning extracts was 60-100 mesh (Fluorisil®, J.T. Baker) and was heat-activated at 105 °C for 48 h. The magnesium silicate was then cooled and stored in a desiccator. Anhydrous sodium sulphate was supplied by *J.T. Baker*. Powdered copper (1 μm) 99% was purchased from Sigma Aldrich. The copper powder was activated with a 10% HNO_3 solution and consecutively rinsed first with distillated water and then with acetone; prior to use, it was dried by an air current. Lauric acid (99.9%) used for standardizing *Fluorisil®* was supplied by *Fluka*. 4,4-dibromobyphenyl (>98%) used as a surrogate was purchased from *Sigma Aldrich*. Benzo[*a*]antracene d-12 obtained from *Cambridge Isotope Laboratories* was used as internal standard.

Sixteen PAHs standards (>98%): indene (Ind), naphthalene (Nap), biphenyl (Byp), dibenzofuran (Dbf), dibenzotiophene (Dbt), acenaphthene (Ace), acenaphthylene (Acy), fluorene (Flu), phenanthrene (Phe), anthracene (Ant), fluoanthene (Flt), pyrene (Pyr), benzo[*a*]anthracene (B[*a*]A), benzo[*a*]pyrene (B[*a*]P), benzo[k]fluoranthene (B[*k*]F) and dibenzo[*a,h*]anthracene (DB[*a,h*]A) were obtained from *Sigma Aldrich*.

Eleven organochlorine pesticides standards (>98%): lindane, heptachlor, aldrin, p,p-DDE, p,p-DDD, p,p-DDT, dieldrin, endrin, mirex, methoxychlor and endosulphan (96% endosulphan I and 4% endosulphan

II); and six organophosphorus pesticides standards (>98%): methyl-parathion, parathion, malathion, phosalone, methyl-azinphos and coumaphos, were obtained from *Alltech*. A permethrin standard (78% cis-permethrin and 20% trans-permethrin) was purchased from *Chem Service*.

2.2. Sample collection and preparation of sediment material

Sediment samples from the confluence of tributary rivers coming from the "Montes Azules" Biosphere Reserve and the Lacantun were collected and transported preserved on ice to the laboratory. Dried, homogenized sediment was used for the optimization and comparison experiments. The sediment was dried at 105°C for 24 hours and then it was crushed and passed through a 2 mm mesh. The main physicochemical properties of the homogenized sediment are presented in Table 1.

Table 1. Physicochemical properties of the homogenized sediment.

Parameter		Parameter	
pH ($CaCl_2$)	7.3	Sand (w/w%)	67.5
TOC[a] (%)	22.5	Slit (w/w%)	28.7
EC (µs)	220	Clay (w/w%)	3.8

[a]Total organic carbon.

2.2.1. Fortification of samples

No more than 300 g of homogenized sediment was deposited in a 1L bowl flask in a rotary evaporator. A sufficient volume of a mixture consisting of acetone/methanol (1/1, v/v) was added to cover the sediment; then, a known amount of standards analyte mixture solution was added in order to obtain an approximate concentration of 50 ng/g for all the analytes on the sediment once the solvent was evaporated. The solvent evaporation was done by maintaining the water bath temperature at 60°C ± 5°C and keeping an intense rotation in order to favor the appropriate homogenization and adsorption of the analytes on the sediment particles. This process takes about 4 to 5 hours to be completed. Finally, the sediment was left for 15 days under room temperature conditions to obtain "aged sediment".

2.3. Extraction procedures

As the extraction optimization and comparison of the extraction procedures is the main objective, it would be discussed in depth in section 2.7. For the evaluation of the best extraction procedure the following techniques were studied: atmospheric pressure focused microwave extraction and ultrasonic bath extraction. Those techniques were compared to the Soxhlet extraction, being this a well know method that is commonly used as comparison standard for other extraction techniques.

2.4. Clean-up of the extracts

Before the clean-up step, the extracts were filtered using qualitative grade filter paper *Whatman #4,* they were then placed on an 40°C ± 5°C water bath and were evaporated to a 2-3 mL volume with a gentle N_2 current. The clean-up consisted in the removal of molecular sulfur and other polar injector reactive compounds that would be removed with the Fluorisil® cartridge.

Sulphur removal was performed by adding to the extract 2 g of activated powdered copper and shaking it for 1 minute. The extract was filtered and concentrated to 1 mL with a gentle N_2 current.

A 10 mL cartridge with a 2-3 mm fat-free cotton layer at the bottom was filled with 1 g of anhydrous sodium sulphate and 2 g ± 0.1 g of Fluorisil® (Lauric acid value 93.5). The cartridge was conditioned with 10 mL of methanol, then the sediment extract was transferred to the cartridge and was left to soak the adsorbent for 1 minute, after that, the extract was eluted using 15 mL of acetone/dichloromethane (1/1, v/v); finally, the volume was reduced to 1 mL with a gentle N_2 current.

2.5. GC-MS conditions

The extracts were analyzed on a HP-6890 gas chromatograph coupled to a HP-5973 mass selective detector (Agilent Technologies, CA, USA). A 25m x 0.25mm i.d. with a 0.25µm film thickness ZB-5 5%-phenyl-95%-dimethylpolysiloxane capillary column was used. The temperature program was as follows: initial

Table 2. Physicochemical properties of the analytes and the target ions and qualifier ions for the GC-MS.

Compound	MW (g/mol)	Log KOW	Vapor pressure (Pa) 25°C	Water solubility (mg/L) 25°C	R.T. (min)	Target ion (*m/z*)	Qualifier ion (*m/z*)	Qualifier ion (*m/z*)	Qualifier ion (*m/z*)	Qualifier ion (*m/z*)
Indene	116.16	2.9	150	30	3.42	116	115	89		
Naphthalene	128.17	3.3	11	31	4.21	128	129	127		
Biphenyl	154.21	3.98	1.2	6.94	5.31	154	153	155		
Acenaphthylene	152.19	4.07	3.86	3.93	5.83	152	151	153		
Acenaphthene	154.21	3.98	0.59	1.93	6.06	153	153	152		
Dibenzofuran	168.19	3.18	2.32	3.0	6.31	168	139			
Fluorene	166.22	4.18	0.04	1.98	6.84	166	165	167		
Lindane*	290.83	3.3	$5.6x10^{-3}$	7.3	8.29*	183	181	109		
Dibenzothiophene*	184.26	*n.a.*	*n.a.*	1.4	8.29*	184	186	139		
Phenanthrene	178.23	4.45	0.09	1.2	8.53	178	179	176		
Anthracene	178.23	4.45	$2.26x10^{-3}$	0.76	8.63	178	176	179		
Methyl-parathion	263.20	3.0	$2x10^{-4}$	55	9.40	109	125	263	79	93
Heptachlor	373.32	5.44	$4x10^{-2}$	0.056	9.51	100	272	274		
Malathion	330.35	2.75	$3.1x10^{-3}$	148	10.03	173	125	127	93	158
Aldrin	364.91	6.5	$3x10^{-3}$	0.027	10.13	66	263	220		
Parathion	291.3	3.83	$8.9x10^{-4}$	12.4	10.28	109	97	291	139	155
4,4-dibromobiphenyla	312.01	5.47	$6x10^{-5}$	*n.a.*	10.49	312	310	314		
Fluoranthene	202.26	4.9	$6.6x10^{-4}$	0.26	11.04	202	201	203		
Pyrene	202.25	4.88	$3.3x10^{-4}$	0.77	11.52*	202	200	203		
Endosulphan I	406.95	3.13	$8.3x10^{-4}$	0.32	11.55*	195	339	341		
p,p-DDE	318.03	6.51	$8x10^{-4}$	0.12	12.01	246	248	176		
Dieldrin	380.91	3.7	$2.4x10^{-5}$	0.14	12.07	79	263	279		
Endrin	380.91	3.2	$2.67x10^{-5}$	0.24	12.53	67	345	250		
Endosulphan II	406.95	3.13	$8.3x10^{-4}$	0.32	12.80	337	339	341		
p,p-DDD	320.04	6.02	0.18	0.09	12.97	235	237	165		
p,p-DDT	354.49	6.91	$2.5x10^{-5}$	0.006	13.79	235	237	165		
Benzo[*a*]anthracene	228.29	5.61	$2.93x10^{-6}$	0.01	14.82	228	229	226		
Benzo[*a*]anthracene d-12b	240.29	*n.a.*	*n.a.*	*n.a.*	14.82	240	239	241		
Methoxychlor	345.65	5.83	$8x10^{-5}$	0.1	15.09	227	228	152	114	274
Phosalone	367.81	4.01	$1.5x10^{-5}$	1.4	15.65*	182	184	367	121	
Methyl-azinphos	317.32	2.96	$5x10^{-7}$	28	15.72*	160	132	93	104	105
Mirex	545.54	5.28	*n.a.*	0.085	15.88	272	237	274	270	239
Cis-permethrin	391.28	6.1	$2x10^{-3}$	0.2	16.83	183	184	163	165	
Trans-permethrin	391.28	6.1	$2x10^{-3}$	0.2	17.05*	183	184	163	165	
Coumaphos	362.77	3.86	$1.3x10^{-5}$	1.5	17.05*	362	226	210	109	97
Benzo[*k*]fluoranthene	252.31	6.06	$1.28x10^{-8}$	$7x10^{-4}$	17.46	252	253	125		
Benzo[*a*]pyrene	252.31	6.05	$7.46x10^{-7}$	$2x10^{-3}$	18.06	252	253	125		
Dibenzo[*a,h*]anthracene	278.40	6.84	$1.33x10^{-8}$	$5x10^{-4}$	20.39	278	139	279		

[a]Subrogade, [b]Internal standard, *Non-real coelution between the compounds, the target ions and qualifier ions are unique or predominant for a selected compound, *n.a.*: not available.

column temperature of 60°C (held for 1 min), 30°C/min to 150°C, 10°C/min to 220°C (held for 1 min), 10°C/min to 300°C (held for 5 min). Helium was used as carrier gas at a constant flow rate of 1 mL/min. The split/splitless injector was set at 270°C and operated in the splitless mode (purge delay 0.5 min, purge flow 30 mL/min). Splitless injection (1 µL) was performed by an HP-6890 automatic injector (Agilent Technologies, CA, USA). The temperature of the ion source and the mass spectrometer transfer line where maintained at 180°C and 290°C, respectively. The detection was operated in the selective ion monitoring (SIM) mode (electron impact: 70 eV). The target ions and the qualifier ions used for quantification are presented in Table 2. A typical chromatogram of a 500 ng/mL standard analyte mixture obtained under these conditions is presented in Figure 1.

2.6. Validation parameters and quality control

All the standard solutions were prepared in an acetone/dichloromethane (1/1, v/v) mixture. The instrumental calibration curve was made by injecting three times each of the seven different analyte concentrations covering a concentration range between 50 and 1000 ng/mL. The relative area vs. concentration was plotted; benzo[*a*]antracene d-12 was used as the internal standard. A monthly calibration control solution was injected; a maximum variation of 10% on the response was set as a limit for recalibrating the instrument. Permethrin, DDT and endosulphan were quantified as the sum of all their isomers.

The method linearity was evaluated by making dilutions of the fortified sediment with non-fortified sediment in such proportions that, once prepared the samples, the concentrations on the extracts would fall on a range between 50 and an 500 ng/mL, which corresponds to a concentration on the sediment in a range between 5 an 50 ng/g. For the USBE and the FMWE extractions, seven concentration points per triplicate were made; for the Soxhlet extraction only 3 concentration points were prepared, as it was unnecessary to add more points because the technique's standards are well established.

In order to evaluate the limit of detection (*LOD*) and the limit of quantification (*LOQ*), additional calibration curves at lower concentrations were made. The concentration was lowered until the ratio signal/noise was below 3. The *LOD* and *LOQ* were calculated interpolating those calibration curves that included at least

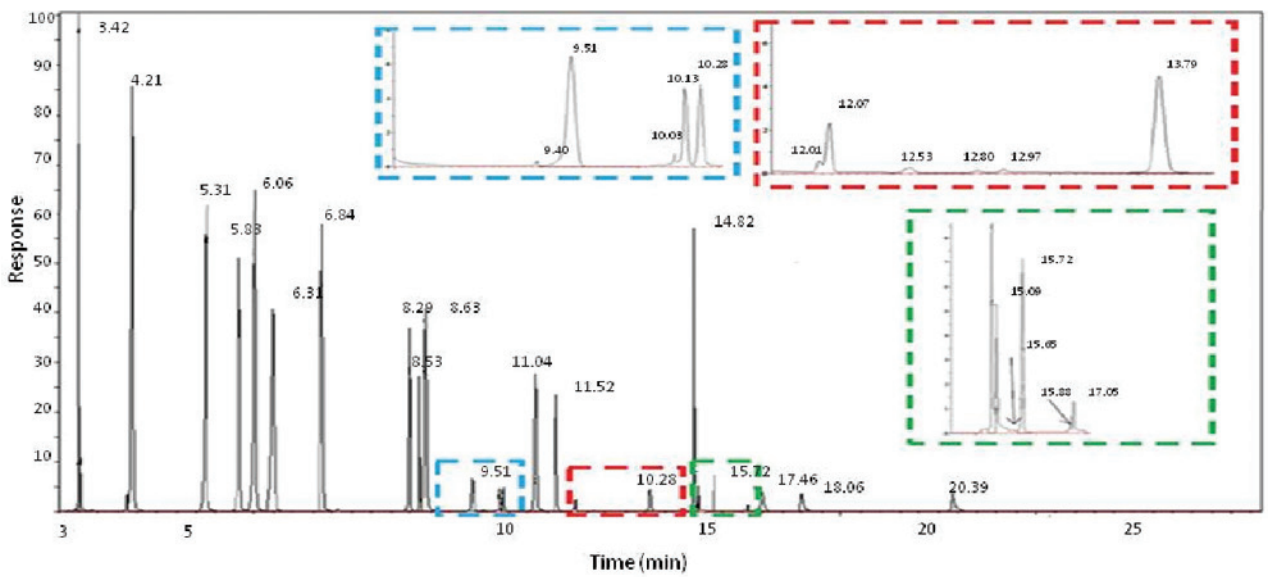

Figure1. Chromatogram of a standard analytes mixture. Concentration: 500 ng/mL.

the five lower concentrations[4]. The *LOD* concentration was interpolated from the calibration curve at a response point at $Y_0 + 3S_0$ while the *LOQ* at $Y_0 + 10S_0$; where Y_0 and S_0 corresponded to the intercept and the standard deviation of the calibration curve. Finally, sediment samples were fortified in order to be in a concentration similar to the *LOD* and *LOQ* once extracted and were analyzed five times; a correct detection and a relative standard deviation below 30% were set as acceptable criteria in order to define the *LOD* and *LOQ* of the method respectively.

The percentage of recovery was evaluated by a surrogate (4,4-dibromobyphenyl); a known amount of this compound was added to each sample before the extraction process. A percentage of recovery between 70% and 130% was the criteria for not rejecting the sample.

Before each batch injection process or every 12 hours the liner reactivity was evaluated. The percentage of degradation of p,p-DDT into its isomers (p,p-DDD and p,p-DDE) was calculated using the following equation:

$$\%\,\mathrm{deg}\,radation \cdot of \cdot DDT = \left(\frac{[area \cdot DDD]+[area \cdot DDE]}{[area \cdot DDD]+[area \cdot DDE]+[area \cdot DDT]}\right)100 \quad (1)$$

When the degradation percent was greater than 15% the liner was replaced.

2.7. Establishment of the extraction conditions and optimization

In order to establish the optimum extraction conditions for the ultrasonic bath and the focused microwave extraction techniques, a series of experiments guided by full factorial and fractional factorial designs were made. The solvents studied were selected according to some official methods and publications[5-20] describing the extraction of a particular group of compounds from soils. Each extraction technique had its particular factors and ranges that were evaluated as is described on the following paragraphs. After each extraction procedure, clean-up of the extracts was performed as mentioned in section 2.3.

2.7.1. Focused microwave extraction

An atmospheric pressure focused microwave extraction system *Prolabo-Maxidigest 350* able to supply 300 W maximum microwave power was used. Ten grams of the prepared sediment were packed in a thimble made of qualitative grade paper filter *Whatman #4*. The cartridge was placed in the extraction tube, the solvent mixture was added, the condenser was attached and the equipment was programmed to follow the respective irradiation cycles. The factors evaluated in this extraction technique were: the number of extractions, the extraction solvent mixture (acetone/hexane 1/1, v/v, acetone/dichloromethane 1/1, v/v), the volume of the extraction solvent mixture, the irradiation time and the non-irradiation period between the irradiation cycles.

2.7.2. Ultrasonic bath extraction

A *Sper Scientific-100005* (50W, 45 KHz) ultrasonic bath was used. Ten grams of the prepared sediment were deposited in a 50 mL glass beaker which was then covered with aluminum paper. The glass beaker was placed on the ultrasonic bath and a sufficient water quantity was deposited into the bath in order to cover tree quarters of the beaker (the water in the bath should be well above the volume of the solvent in the beaker), this condition was maintained constant during all the experiments –this is a critical factor because it affects the ultrasound transmission from the transducer to the sample. The factors evaluated were: the number of extractions, the extraction solvent mixture (acetone/hexane 1/1, v/v, acetone-dichloromethane 1/1, v/v), the volume of the extraction solvent mixture and the irradiation time.

2.7.3. Soxhlet extraction

The Soxhlet extraction was carried out based on the conditions established by[21]. Ten grams of prepared sediment were weighed into the extraction thimble and extracted with 40 mL of acetone-dichloromethane (1/1, v/v) for 6 h at 10-12 cycles per hour.

2.8. Comparison between the extraction techniques

A precisely weighed prepared sediment sample was analyzed five times by each optimized extraction technique. Student t-test, analysis of variance (ANOVA) and the respective post-hoc tests were performed in order to determine significant differences between the extraction techniques.

3. Results and discussion

3.1. Optimization of the extraction techniques

3.1.1. Focused microwave extraction

The focused microwave equipment program can only support the adjustment of increments in 30 W of microwave power and time intervals of one minute; this is due to the fact that the total irradiation time corresponds to the number of irradiation cycles for one minute at 30 W of microwave power applied to the sample. For each experiment the first irradiation minute was done with a 60 W microwave power output in order to elevate the mixture temperature close to its boiling point after the first irradiation cycle. A microwave power of 30 W was set in order to bring the solvent mixture near boiling point, higher power outputs caused solvent projections and lose of sample.

Considering there were 5 defined factors for this extraction technique, performing a 2^5 factorial design would result in making 32 experiments and even if this design has a maximum resolution between the factors, it cannot assure that the optimum conditions would be in the selected factor ranges, so performing such a high resolution factorial design is not appropriate for screening purposes due to the waste of time and laboratory resources. For screening purposes, lower resolution designs should be used. Even if they have confounded the interaction factors responses, they are able to discern between the responses of the principal factors. This extraction technique was developed in three factorial designs, the factors and the ranges evaluated are summarized in Figure 2.

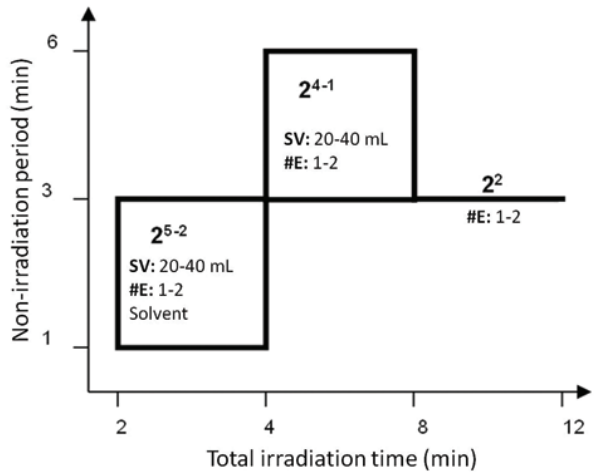

Figure 2. Factorial designs carried out in order to establish the extraction conditions using focused microwaves. The factors and ranges evaluated for a specific design are presented. SV: extraction solvent volume. #E: number of extractions.

After performing the experimental design, the amount extracted of each analyte was calculated. The effect of the factors and interactions for each analyte was calculated with the following equation:

$$factor \cdot efect = \left(\frac{\mathrm{E}_{i=1}^{n} C^{+}}{n} \right) - \left[\frac{\mathrm{E}_{i=1}^{n} C^{-}}{n} \right] \quad (2)$$

where C^+ and C^- correspond to the measured concentration of an analyte at the higher and lower factor level of the corresponding experiments, respectively. The most significant factors for each analyte could thus be identified. However, the main purpose was to identify the best extraction conditions for all the analytes; in order to do so, the desirability index for each experiment was calculated using the following equation:

$$D = \left(d_1 \bullet d_2 \bullet .... d_m \right)^{1/m} \quad (3)$$

where d_i corresponds to the individual desirability index of an analyte in a particular experiment and m to the total number of observations. The individual desirability index varies between a range of $0 \leq d_i \geq 1$, the value of 1 was assigned to the highest concentration in the design experiments for a specific analyte, besides, a value of 0 was assigned to the lowest concentration; relative to the

highest and to the lowest concentrations the values of the individual desirability indexes for an analyte were calculated. With these, the desirability index for each experiment that involved a particular combination of factor levels could be observed.

The first design performed was a 2^{5-2} fractional factorial design; this design was done in 8 experiments -the combinations of the factor levels and desirability indexes are shown in Table 3. As can be observed, the highest desirability indexes are obtained when using acetone/dichloromethane (1/1, v/v) solvent mixture and two extractions, the lower indexes can be observed in all the cases in which the non-irradiation period was 1 minute. The solvent mixture shows a high effect over the extraction of PAHs and pesticides due to the difference between the dipolar moment of hexane (0.8 D) and dichloromethane (1.14 D). Dichloromethane shows a better solubilizing effect over pesticides since it has more affinity to their polarity and structure when compared to hexane, which predominantly shows good results for the extraction of PAH´s. Higher numbers of extractions improve the extraction recoveries because the extraction equilibrium is displaced; however this effect can be replaced by performing higher irradiation times, as was demonstrated in the next designs. In the surface response plot of this design (Figure 3) we can observe

Table 3. Combination of factor levels and the obtained desirability indexes for the 2^{5-2} design.

Experiment	Factor					Desirability index
	A	B	C	D	E	
1	-	-	-	+	+	0.25
2	+	-	-	-	-	0.45
3	-	+	-	-	+	0.96
4	+	+	-	+	-	0.82
5	-	-	+	+	-	0.25
6	+	-	+	-	+	0.25
7	-	+	+	-	-	0.11
8	+	+	+	+	+	0.76

A: total irradiation time (2-4 min). B: non-irradiation period between irradiation cycles (1-3 min). C: Extraction solvent mixture ([+] acetone/hexane 1/1, v/v) and ([-] acetone/dichloromethane 1/1, v/v). D: volume of the solvent extraction mixture (20-40 mL). E: number of extractions (1-2).

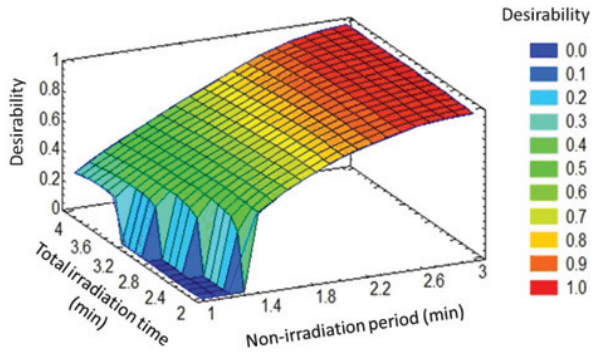

Figure 3. Surface response plot for the 2^{5-2} design of the focused microwave extraction. The desirability index as function of the non-irradiation period and the total irradiation time maintaining constant 40 mL solvent extraction volume, two extractions and acetone/dichloromethane (1/1, v/v) as extracting solvent is plotted.

that irradiation periods of one minute are not favorable as the solvent cannot be cooled fast enough and in the next irradiation cycle the temperature reaches the boiling point of the solvent mixture producing explosions and loses of the analytes.

As the best solvent mixture consists of acetone/dichloromenthane (1/1, v/v) the rest of the experiments were performed using this mixture. A second design of higher resolution (2^{4-1}) was made, the desirability indexes and combinations of the factor levels can be observed in Table 4. Incrementing the irradiation time is favorable for improving the extraction; the opposite effect is observed when the non irradiation period is greater than 4 minutes, where the solvent mixture reaches a temperature that is not close to the boiling point in the next irradiation cycle. Maintaining these conditions has a considerable impact on the extraction efficiency, as can be observed in Figures 4 and 5. There is not a high difference between the desirability indexes when two extractions using a volume of 20 mL (Figure 3) and 40 mL (Figure 5) of extraction solvent are compared; by performing the experiment with only one extraction with 40 mL of extraction solvent the desirability index is significantly lower (graph not shown). This effect is logical as it is well known that incrementing the number of extractions displaces the extraction equilibrium.

Table 4. Combination of factor levels and the obtained desirability indexes for the 2^{4-1} design.

Experiment	Factor				Desirability index
	A	B	C	D	
1	-	-	-	-	0.27
2	+	-	-	+	0.68
3	-	+	-	+	0.30
4	+	+	-	-	0
5	-	-	+	+	0.57
6	+	-	+	-	0.87
7	-	+	+	-	0.51
8	+	+	+	+	0.51

A: total irradiation time (4-8 min). B: non-irradiation period between irradiation cycles (3-6 min). C: number of extractions (1-2). D: volume of the solvent extraction mixture (20-40 mL).

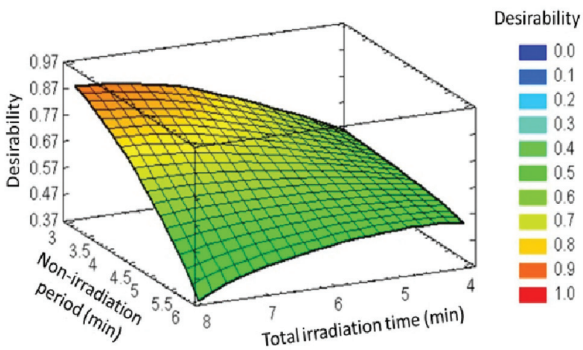

Figure 4. Surface response plot for the 2^{4-2} design of the focused microwave extraction. The desirability index as function of the non-irradiation period and the total irradiation time maintaining constant 20 mL solvent extraction volume, two extractions and acetone/dichloromethane (1/1, v/v) as extracting solvent is plotted.

With this experimental design, two more factors can be defined: a non-irradiation period of 3 minutes and two extractions using a 20 mL volume of the extraction solvent, resulting more suitable for the reduction of the amount of organic solvents used. However, the hypothesis that with higher irradiation periods the extraction could be improved remains, suggesting that one simple extraction is needed. This was studied using a complete 2^2 factorial design in which the evaluated factors were the irradiation period in a range between 8 and 12 minutes and the number of extractions between one and two. The surface response plot (Figure 6) demonstrates that raising the irradiation time does not have an effect over the extraction performance, so there is no sense in investing more time performing longer extractions that could lead to the degradation of some analytes, also the difference between using one or two extractions is more evident with this design as it exposes a high resolution between the factors.

By these series of experiments the optimum experimental extraction conditions were established: 9 minutes of irradiation time, the first minute at a 60 W power output and the rest at 30 W; a non-irradiation period of 3 minutes; and two consecutive extractions using 20 mL of acetone/dichloromenthane (1/1, v/v) as extraction solvent. In Figure 7 the extraction time vs. irradiation power and the bulk solvent temperature is

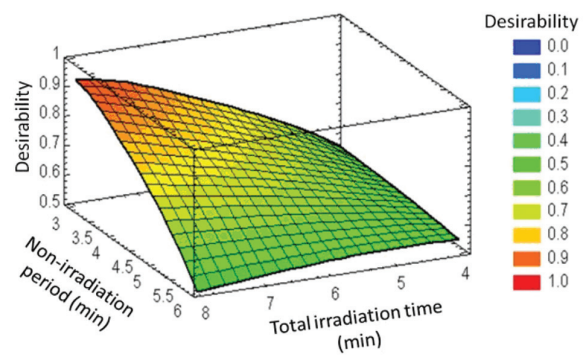

Figure 5. Surface response plot for the 2^{4-2} design of the focused microwave extraction. The desirability index as function of the non-irradiation period and the total irradiation time maintaining constant 40 mL solvent extraction volume, two extractions and acetone/dichloromethane (1/1, v/v) as extracting solvent is plotted.

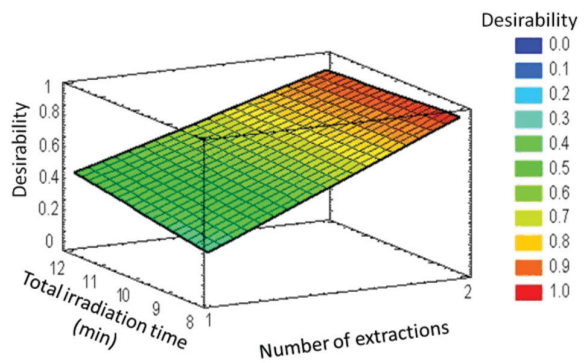

Figure 6. Surface response plot for the 2^2 design of the focused microwave extraction. The desirability index as function of the total irradiation time maintaining constant the non-irradiation period at 3 minutes, 20 mL solvent extraction volume, two extractions and acetone/dichloromethane (1/1, v/v) as extracting solvent is plotted.

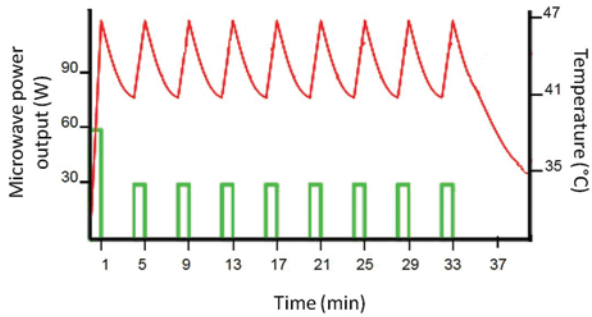

Figure 7. Focused microwave extraction cycle. The time vs. the irradiation power and the bulk solvent temperature of the optimized extraction method is plotted.

plotted. One extraction is carried in 33 minutes so the overall extraction process for one sample is carried out in about 90 minutes considering cooling and handling. The solvent temperature is maintained at a constant range during the extraction between 41°C and 47°C as shown in Figure 7; the solvent temperature is kept from reaching boiling point because that would result in partial analytes loss.

3.1.2. Ultrasonic bath extraction

Based on the results observed on the focused microwave extraction, a mixture of acetone/dichloromethane (1/1, v/v) was used in all the experiments for the optimization of the ultrasonic bath extraction. The three remaining factors -number of extractions, solvent volume and irradiation time- were evaluated by a 2^3 factorial design. The evaluated ranges of the irradiation time and the extraction volume were established in a ± 50% range of the maximum and minimum parameters set in previous works for some group of compounds[13,16-19]; also, two to four extractions were evaluated based on previous experiences and on the well known enhancement of the extraction by means of a higher number of consecutive extractions. As the factor ranges for this extraction technique were greater than those for the focused microwave extraction, 4 central points were added to the factorial design study in order to increment the precision for the discrimination of significant factors. As the ultrasonic bath extraction has more variability due to the multiple factors that affect the ultrasonic energy transmission[22-24], this design was performed in four blocks of three experiments in order to evaluate the variability between days. The desirability indexes for the experiments are shown on Table 5. The higher desirability indexes are observed when four consecutive extractions with 30 mL of solvent are used; the desirability index is a little higher when 3 minutes of irradiation is applied; however, once the surface response plot is generated, the optimum conditions are observed after 10 minutes of irradiation. This difference in the desirability index between the irradiation time is small enough that the extraction could be done at any irradiation period without a high variability on the results, as can be observed on Figure 8 where a practically constant desirability index is obtained when the solvent volume is near 30 mL. The surface response plot demonstrates that there is a considerable robustness for some parameters in the extraction; this orients the analyst as to where the focus in future extractions should be placed. The solvent volume has a critical role over the extraction because it acts as a medium of transmission for the ultrasonic energy applied to the sample; a volume barely higher than that of the sample volume does not allow efficient

Table 5. Combination of factor levels and the obtained desirability indexes for the 2^3 design.

Experiment	Block	Factor			Desirability index
		A	B	C	
1		*	*	*	0.56
2	1	-	+	+	0.92
3		+	-	-	0.35
4		*	*	*	0.65
5	2	+	+	-	0.40
6		-	-	+	0.60
7		-	+	-	0.40
8	3	*	*	*	0.64
9		+	-	+	0.81
10		-	-	-	0.19
11	4	+	+	+	0.90
12		*	*	*	0.56

A: irradiation time (3-15 min). B: volume of the solvent extraction mixture (15-30 mL). C: number of extractions (2-4). *Denotes a central point of the experimental design, the value of that factor is the center value of the factor range.

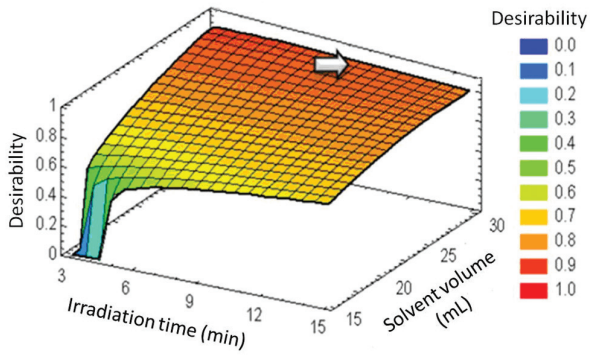

Figure 8. Surface response plot for the 2^{4-2} design of the focused microwave extraction. The desirability index as function of the irradiation time and the solvent volume, maintaining constant four extractions and acetone/dichloromethane (1/1, v/v) as extracting solvent is plotted. The optimum experimental selected conditions are marked with an arrow.

ultrasonic energy transmission ; this results in a poor diffusion of the analytes into the medium, as can be read from Figure 8. The selected experimental conditions for this extraction were: 30 mL of acetone/dichloromethane (1/1, v/v) as extracting solvent; 10 minutes of irradiation time; and four consecutive extractions.

3.2. Validation parameters

3.2.1. Instrumental and method linearity

In all the cases the instrumental linearity was acceptable when a determination coefficient (*R*) greater than 0.98 (**data not shown**) was obtained. In the method linearity, most of the analytes showed a determination coefficient greater than 0.98; however, some others presented lower determination coefficients, but in all the cases they were higher than 0.70 (**data not shown**), which was set as the lowest acceptable value due to the sample and method variability, this criterion was taken from the Horwitz trumpet graph[25] that establishes the maximum accepted relative standard deviation (*RSD*) as function of the sample concentration; for our working concentrations, the *RSD* should be lower than 30%. All the method calibration curves were compared to the instrumental calibration curves in order to evaluate the effect of the matrix interferences. An ideal behavior was observed for most of the cases in which the method calibration curves had a similar or lower slope as compared with the instrumental calibration curve (this can be observed for *benzo[a]anthracene* in Figure 9). A lower slope of the method calibration curve than that of the instrumental calibration curve is indicative of a lower extraction efficiency; however, this behavior could also be attributed to the loss of the analytes during the fortification process, due to volatilization during the aging period or to irreversible adsorptions over the sediment components. A matrix response suppression was discarded because of the universal character of the detector. On the other hand, a matrix interference was observed as a higher response of the method calibration curve, this was observed for *methyl-parathion* (Figure 10) and *phosalone* (graph not shown) using focused microwave extraction, thus, for this technique those compounds were not included on the comparative

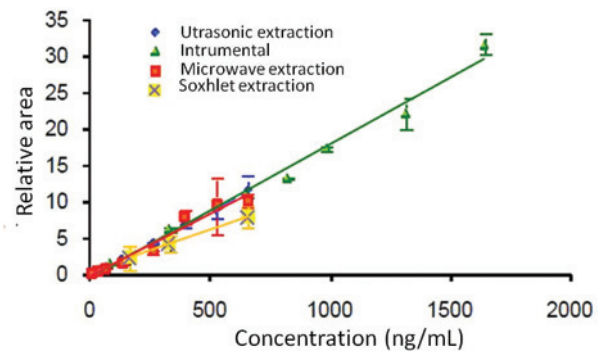

Figure 9. Instrumental and method calibration curves for *benzo[a] anthracene*.

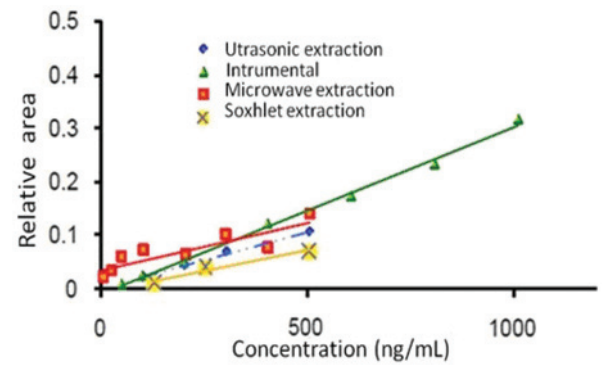

Figure 10. Instrumental and method calibration curves for *methyl-parathion*.

study. It can be observed on Figure 10 that using only microwave extraction some interferences were extracted, this effect is significant at lower analyte concentration levels. Despite that all the analytes had a determination coefficient greater than 0.7, comparing the calibration curves evidenced that *methyl-parathion* and *phosalone* are interfered, so plotting the method curves should be necessary to demonstrate the absence of interfering compounds; to determine an adequate working interval; and to verify the percentages of recovery at different concentration levels.

3.2.2. LOD and LOQ

Because USBE turned out to be the most versatile extraction technique for all the analytes, only the corresponding *LOD* and *LOQ* are presented, (**see section 3.3**). As can be seen in Table 6, the PAH´s present significantly lower *LOD* and *LOQ* than the pesticides, this behavior is attributable to the lower fragmentation of the molecule in the mass spectrometer, due to the higher stability of the conjugated rings of the PAH´s, which generate a higher number of fragments of a specific *m/z* relation (molecular ion in the case of PAH´s); thus, the total ion current is reduced for these species.

3.3. Comparison between the extraction techniques

An ANOVA test was performed to the sample concentrations of the evaluated extraction techniques; previously, the hosmoscedasticity of the data was demonstrated using *Levene's test*[26]. For the analytes in which ANOVA suggested a significant difference between the means, *Fisher's least significant difference* (LSD) post-hoc test was performed[27]. Based on the statistical results, for each analyte, a value of 100% was assigned to those which presented the highest concentration obtained in the evaluated extraction techniques; the rest of the analytes evaluated by other extraction techniques were assigned a percentage relative to the maximum of each type. Figure 11 presents the relative extraction efficiencies of the techniques for each analyte. By these, it could be easy to identify the

Table 6. *LOD* and *LOQ* for the ultrasonic bath extraction method.

Compound	*LOD* (ng/g)	*LOQ* (ng/g)	Compound	*LOD* (ng/g)	*LOQ* (ng/g)
Lindane	1.1	3.8	Indene	0.02	0.05
Heptachlor	0.8	2.6	Naphtalene	0.02	0.06
Aldrin	0.6	1.9	Bypehenyl	0.01	0.02
Dieldrin	0.6	2.0	Acenaphthylene	0.01	0.02
Endrin	1.5	4.9	Acenaphthene	0.01	0.02
Mirex	1.4	4.7	Dibenzofurane	0.01	0.05
Methoxychlor	0.4	1.3	Fluorene	0.01	0.05
p,p-DDT	0.4	1.3	Dibenzothyophene	0.01	0.02
p,p-DDE	0.5	1.6	Phenanthrene	0.004	0.01
p,p-DDD	1.1	3.7	Anthracene	0.005	0.02
Methyl-parathion	1.4	4.5	Fluoranthene	0.01	0.04
Parathion	2.6	8.7	Phyrene	0.02	0.05
Malathion	1.7	5.6	Benzo[*a*]anthracene	0.02	0.06
Phosalone	0.7	2.3	Benzo[*k*]fluoranthene	0.03	0.06
Methyl-azinphos	11.0	36.0	Benzo[*a*]phyrene	0.06	0.2
Coumaphos	29.0	97.0	Dibenzo[*a,h*]anthracene	0.005	0.16
Permethrin	0.95	3.1			

LOD: limit of detection; *LOQ*: limit of quantification. $n = 5$, *RSD* < 30%.

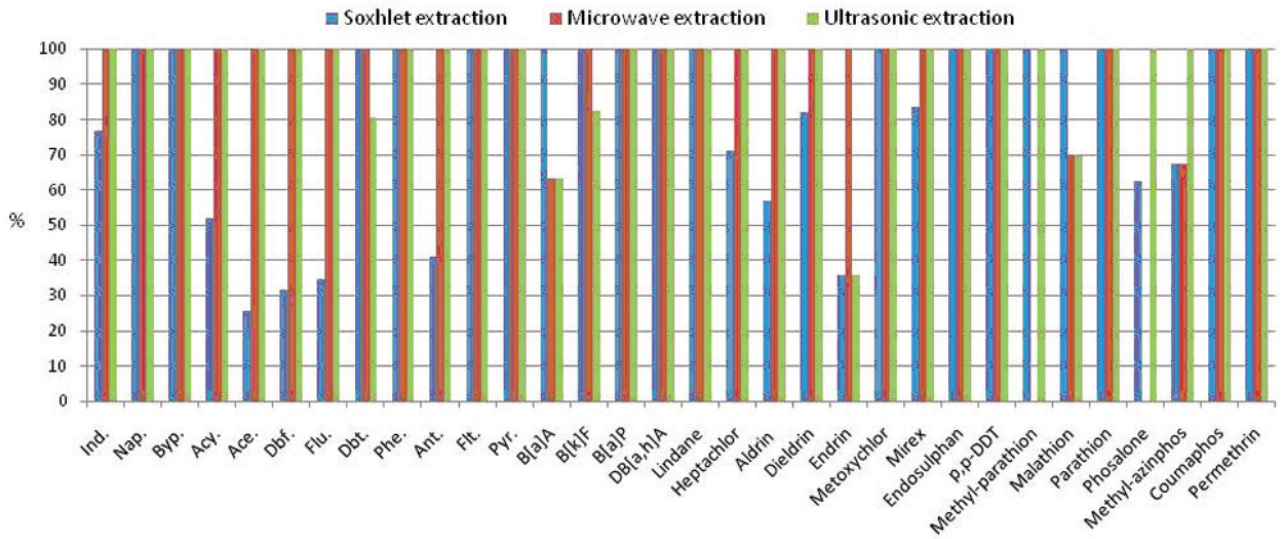

Figure 11. Relative extraction efficiencies of the evaluated techniques for each analyte. A value of 100% is assigned to the highest extracted concentration of the analyte in the evaluated techniques; the remaining percentages are assigned in relation to the best one. Phosalone and Methyl-parathion were not included using microwave extraction due to the extraction of interferences.

best extraction technique for a specific analyte; however, most of the analysis are multicomponent so it is more convenient to visualize the extraction efficiencies including all the analytes.

Based on the results presented in Figure 11, the relative extraction efficiency per group of compounds can be calculated (Figure 12). Five groups of compounds were established: the organophosphorus pesticides, the organochlorine pesticides, the piretroid pesticides and the HAP´s, this last was divided into two groups; the low molecular weight PAHs (MW<170 g/mol) and the PAHs with a MW>170g/mol. This subdivision between the PAHs was made as consequence of the experimental results, the PAH´s whith a MW<170g/mol are more volatile than the rest of the PAH´s which are less volatile and more lipophilic. As can be seen in Figure 12, Soxhlet extraction is less efficient for PAH´s with MW<170g/mol, due to the high volatility of these compounds. It is convenient to remember that most of these compounds have the ability to sublime at laboratory conditions. The remaining PAH´s can be extracted either by focused microwave or ultrasonic bath, showing slightly better results in the focused microwave extraction. The organochlorine pesticides are best extracted using focused microwave;

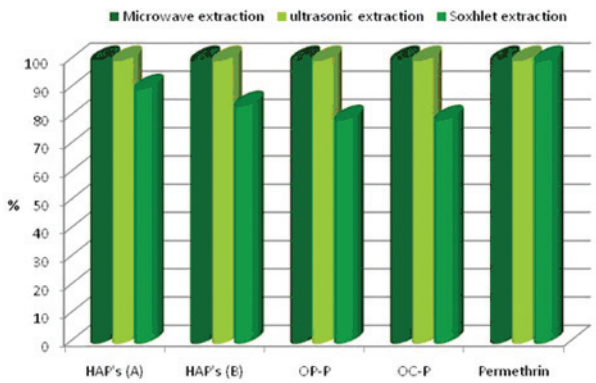

Figure 12. Relative extraction efficiencies of the evaluated techniques for the groups of compounds. (A): PAH´s with a molecular weight below 170g/mol. (B): PAH´s with a molecular weight above 170 g/mol. OP-P: organophosphorus pesticides. OC-P: organochlorine pesticides.

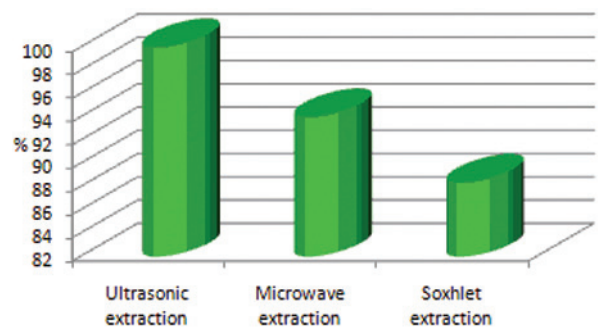

Figure 13. Relative extraction efficiencies of the evaluated techniques for all the analytes.

Table 7. Consumed time during the sample preparation by three extraction techniques.

	Ultrasonic extraction		Microwave extraction		Soxhlet extraction	
# of Samples	1	6	1	6	1	6
Extraction	40 min	40 min	80 min	480 min	360 min	2160 min
Clean-up	10 min	60 min	10 min	60 min	10 min	60 min
Concentration	180 min	180 min	120 min	120 min	90 min	90 min
Total time	3.8 h	4.7 h	3.5 h	11 h	7.7 h	3.8 h

however, the ultrasonic bath extraction presents only a 10% lower efficiency, which could be increased using a more powerful ultrasonic bath or a ultrasonic probe which can supply 400 W. Focused microwave extraction is not very recommendable for the organophosphorus pesticides; it was determined that the extraction efficiency is 40% lower compared to the other extraction techniques. This behavior could be accounted to the rise in the degradation kinetics of these analytes, which are known to hydrolyze more easily than other pesticides or to favor irreversible adsorptions with the polar groups of the humic-acids present in sediments. Finally it can be observed that *permethrin* can be extracted by any technique.

Taking into account the relative extractions of all the analytes, a relative extraction efficiency per extraction technique can be established. It can be seen in Figure 13 that the ultrasonic bath extraction has the highest results; nevertheless, the focused microwave and the Soxhlet extraction only presented 8% and 14% lower efficiencies respectively, which could be non-significant for environmental purposes at low concentration levels. Analyzing other steps in the sample preparation such as the clean-up and the concentration of the extract, it can be observed (Table 7**)** that the fastest technique for analyzing one sample is the focused microwave; however, for environmental monitoring in which the number of samples may be very large, the advantage of using an ultrasonic bath is evidenced. Considering six samples (maximum ultrasonic bath capacity) the overall sample preparation time can be done in less than 5 hours, while with the focused microwave and the Soxhlet extraction the sample preparation is performed in up to eleven and forty hours respectively.

4. Conclusions

The development and optimization of the simultaneous extraction of PAH´s and pesticides was done using factorial designs. These are helpful in establishing the extraction conditions when numerous factors are involved; simultaneously evaluating the interaction between the selected factors cannot be done with so few experiments when analyzing one variable at a time; by means of these, time and resource investment lower considerably, and there is a high level of confidence that the optimum extraction conditions can established. The methods using the extraction techniques meet the validation criteria by showing determination coefficients above 0.7 in all cases and above 0.98 in 90% of the selected analytes, the RSD was below 30% in all cases and the percentage of recovery was between 70% and 130%. In focused microwave extraction, compounds that interfered with the detection of phosalone and methyl-parathion were also extracted; however, this can be solved by modifying the chromatographic conditions.

The microwave extraction and the ultrasonic extraction did not show a statistically significant difference on the extraction of PAH´s with MW<170g/mol; the Shoxhlet extraction presented an efficiency 40% lower. For the rest of the PAH´s (MW>170g/mol) there are no great differences between the extraction techniques. For the organochlorine pesticides the microwave extraction produced the best results, while the ultrasonic and the Soxhlet extraction have efficiencies 8% and 20% lower. The ultrasonic extraction gave the best results for the organophosphorus pesticides, whereas the microwave extraction and the Soxhlet extraction

showed efficiencies 8% and 40% lower. There was no significant statistical difference between the evaluated extraction techniques for *permethrin*.

For the extraction of all the analytes, the extraction technique with the best efficiencies was the ultrasonic extraction. Additionally, it is also the most versatile technique due to its easy handling and fast extraction when many samples are analyzed; also, it implies low operation costs and does not require an unconvenient amount of laboratory space.

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O Bookstore divulgará o material de informação e formação produzido pelo IIC, incluindo Livros, filmes, slides, material didático de cursos e outras formas de divulgação dos materiais por ele produzido. Esses materiais podem ser adquiridos do IIC através do website www.iicweb.org/biblioteca.php

Cromatografia Líquida Moderna - Fernando M. Lanças

Livro publicado pela Editora Átomo em maio de 2009, aborda de forma simples e didática os principais assuntos relacionados à Cromatografia Líquida Moderna (HPLC ou CLAE), desde a Teoria, Instrumentação, Colunas, Detectores, até aspectos da Validação, Preparo da Amostra e Análise Quantitativa.

O IIC recomenda este livro para todos os interessados em iniciar-se ou atualizar-se nesta técnica.

Cromatografia em Fase Gasosa - Fernando M. Lanças

Livro de autoria do Prof. Lanças, abordando todos os princípios básicos: instrumentação, análise qualitativa e quantitativa, detectores e muitos outros aspectos da Cromatografia Gasosa.

Altamente recomendado para os iniciantes na técnica, os quais encontrarão explicações detalhadas e simples a respeito da maior parte dos fundamentos básicos da técnica.

Validação de Métodos Cromatográficos - Fernando M. Lanças

Livro de autoria do Prof. Lanças, descreve os princípios da validação de métodos cromatográficos com detalhes. Enfoque também a validação de instrumentação e a adequação dos sistemas ("*system suitability*") frente aos requisitos dos órgãos regulamentadores de resultados analíticos. O livro é acompanhado de um software, denominado Validate – versão demonstração – desenvolvido em colaboração com o Dr. Vitor Hugo P. Pacces, o qual auxilia no processo de validação.

Extração em Fase Sólida - Fernando M. Lanças

De autoria do Prof. Lanças, este livro apresenta e discute as várias formas de extração em fase sólida (SPE), desde a mais clássica empregando cartuchos até as mais atuais como discos, placas, ponteiras, e outras. Também discute os princípios da micro extração em fase sólida (SPME) e da extração por sorção em barras de agitação (SBSE).

Código da Disciplina	Mês	Data	Nome da Disciplina	Carga Horária
LC-01	Março	26 a 28	Cromatografia Líquida Básica – (HPLC/CLAE)	24
GC-01	Abril	09 a 11	Cromatografia Gasosa Básica – (HRGC)	24
SP-01	Maio	07 a 09	Técnicas Modernas de Preparo de Amostras	24
LC-MS-01	Junho	24 a 27	Acoplamento LC-MS e LC-MS/MS	32
Especial	Ag - SIMCRO		Técnicas Modernas em HPLC	8
Especial	Ag - SIMCRO		Técnicas Modernas em HRGC	8
Especial	Ag - SIMCRO		Técnicas Modernas de Preparo de Amostras	8
Especial	Ag - SIMCRO		Acoplamento LC-MS/MS	8
GC-MS-01	Setembro	23 a 26	Acoplamento GC-MS e GC-MS/MS	32
LC-MS-01	Outubro	21 a 24	Acoplamento LC-MS e LC-MS/MS	32
CRO-00	Novembro	11 a 14	Técnicas Cromatográficas de Análise	32
LC-01	Novembro	26 a 28	Cromatografia Líquida Básica – (HPLC/CLAE)	24

CURSOS COM TEORIA E PRÁTICA

Os cursos promovidos pelo IIC são ministrados por docentes e convidados, e abordam vários temas de grande relevância no momento. Consulte-nos em relação à valores e descontos especiais, bem como sobre transporte e hospedagem.

ISHT

"13th Int. Symp. on Hyphenated Techniques in Chromatography and Separ. Technol. & 3rd Int. Symp. on Hyphenated Techniques for Sample Preparation"
28-31 Jan 2014
Bruges, Belgium
Information: www.htc-conference.org

ICACE

"International Conference Advances in Chromatography and Electrophoresis & Chiranal 2014"
10-14 Feb 2014
Olomouc, Czech Republic
Information:http://chiranal2014.upol.cz

ICFCS

"4th Int. Conf. of the Flow Chem. Soc."
18-19 Feb 2014
Barcelona, Spain
Information: http://selectbio.com/FCE2014

Pittcon 2014

"Pittcon 2014, Conference & Expo"
2-6 March 2014
Chicago, IL, USA
Information: http://www.pittcon.org

MSB 2014

"MSB 2014, 30th Int. Symposium on Microscale Bioseparations"
27 Apr - 1 May 2014
Pécs, Hungary
Information: www.msb2014.org

Prep 2014

"27th International Symposium, Exhibit and Workshop on Preparative LC"
20-23 Jul 2014
Boston, USA
Information: http://www.PREPsymposium.org

ISMS

"20th International Mass Spectrometry Conference"
24-29 Aug 2014
Geneva, Switzerland
Information: http://www.imsc2014.ch/

ISSS 2014

"20th International Symposium on Separation Sciences"
30 Aug 2014 - 02 Sep 2014
Czech Republic
Information: http://www.isss2014.cz/en/

ISHPOP

"34th International Symposium on Halogenated Persistent Organic Pollutants"
31 Aug 2014 - 05 Sep 2014
Madrid, Spain
Information: http://www.dioxin2014.org/

JASIS 2014

"Discover the Future". Analytical Instruments & Solution Expo/ Scientific Instruments Show
03-05 Sep 2014
Japan
Information: http://www.jasis.jp/2014/index.html

SETAC 2014

SETAC ("Society of Environmental Toxicology and Chemistry") Asia Pacific and Australasian 2014 Conference.
14-17 Sep 2014
Adelaide, SA., Australia
Information: http://www.setac.org/

ISC 2014

30th International Symposium on Chromatography
14-18 Sep 2014
Salzburg, Austria
Information: http://www.isc2014.at/

Polyolefin Characterization

"5th International Conference on Polyolefin Characterization"
21-24 Sep 2014
Valencia, Spain
Information: http://www.icpc-conference.org/

Analytica China 2014

"7th International Trade Fair for Laboratory Technology, Analysis, Biotechnology and Diagnostics"
Shanghai New International Expo Centre (SNIEC)
24-26 Sep 2014
Shanghai, China
Information: http://www.analyticachina.com/

World of Technology and Science

"Visiting World of Technology & Science is free"
30 Sep 2014 - 03 Oct 2014
The Netherlands
Information: http://wots.nl/

India Chem 2014

"8th International Exhibition & Conference"
09-20 Oct 2014
Mumbai, India
Information: http://www.indiachem.in/

WWEM 2014

"Water, Wastewater & Environmental Monitoring"
05-06 November 2014
Conference
Telford, UK
Information: www.wwem.uk.com

India Lab 2014

06-08 Nov 2014
Hyderabad, India
Information: http://www.indialabexpo.com/

ISCC GCxGC 2015

"39th International Symposium on Capillary Chromatography and 12th GCXGC Symposium"
17-21 May 2015
Information: http://www.isccgcxgc2015.com/

63rd ASMS Conference

"Conference on Mass Spectrometry and Allied Topics".
31 May 2015 - 04 Jun 2015
St Louis, USA
Information: http://www.asms.org/

HPLC 2015

"42nd International Symposium on High Performance Liquid Phase Separations and Related Techniques"
21-25 Jun 2015
Geneva, Switzerland
Information: http://www.imsc2014.ch/

PEFTEC 2015

"Monitoring and analytical technologies for the Petroleum, Refining and Environmental Industries".
18-19 Nov 2015
Antwerp, Belgium
Information: http://www.peftec.com/

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Escopo

Scientia Chromatographica publica trabalhos em todas as áreas da Cromatografia, incluindo **GC** (colunas capilares, empacotadas, preparativas), **LC** (convencional, HPLC, U-HPLC, Prep-LC, micro-LC, nano-LC, TLC, PC), **SFC** (colunas empacotadas ou capilares), **Técnicas Acopladas** (GC-MS, LC-MS, SFC-MS, LC-GC, SFE-CE, GCxGC, LCxLC) e **Técnicas de Preparo de Amostras** (SPME, SBSE, MEPS, QuEChERS, LLE, MAE,SPE, LLE, etc.). A partir de 2012 o *Scientia* publica os seguintes formatos de artigos:

- Artigos Originais de Pesquisa
- Comunicação ("Short Communication")
- Artigos de Revisão Crítica

Os artigos de Revisão Crítica deverão antes de sua submissão serem discutidos com um dos co-editores mais relacionados ao assunto em questão. Devem conter uma contribuição nova a respeito de um assunto de grande interesse atual, incluindo uma discussão a respeito das vantagens e desvantagens do mesmo. O *Scientia* não publica apenas relato de artigos descritos na literatura, sem uma discussão crítica sobre os mesmos.

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Os artigos do tipo Comunicação deverão ter a mesma qualidade dos artigos Originais, porém devido a seu caráter de comunicação preliminar usualmente são de menor extensão.

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Artigos de Revisão crítica: revisões críticas sobre uma area específica das técnicas cromatográficas e relacionadas são também consideradas para publicação. Esses artigos devem se limitar a no máximo 10.000 palavras, incluindo as legendas, e até 10 tabelas e figuras **somadas**. Os artigos de Revisão Crítica deverão antes de sua submissão serem discutidos com um dos co-Editores mais relacionados ao assunto em questão. Artigos não encaminhados desta forma serão antes enviados a um co-Editor da área em que se enquadre para parecer e, somente então, serão enviados aos revisores, podendo atrasar significativamente sua publicação. Devem conter uma contribuição nova a respeito de um assunto de grande interesse atual, discutindo as vantagens e desvantagens do mesmo. O *Scientia* não publica apenas relato de artigos descritos na literatura, sem uma discussão crítica sobre os mesmos.

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