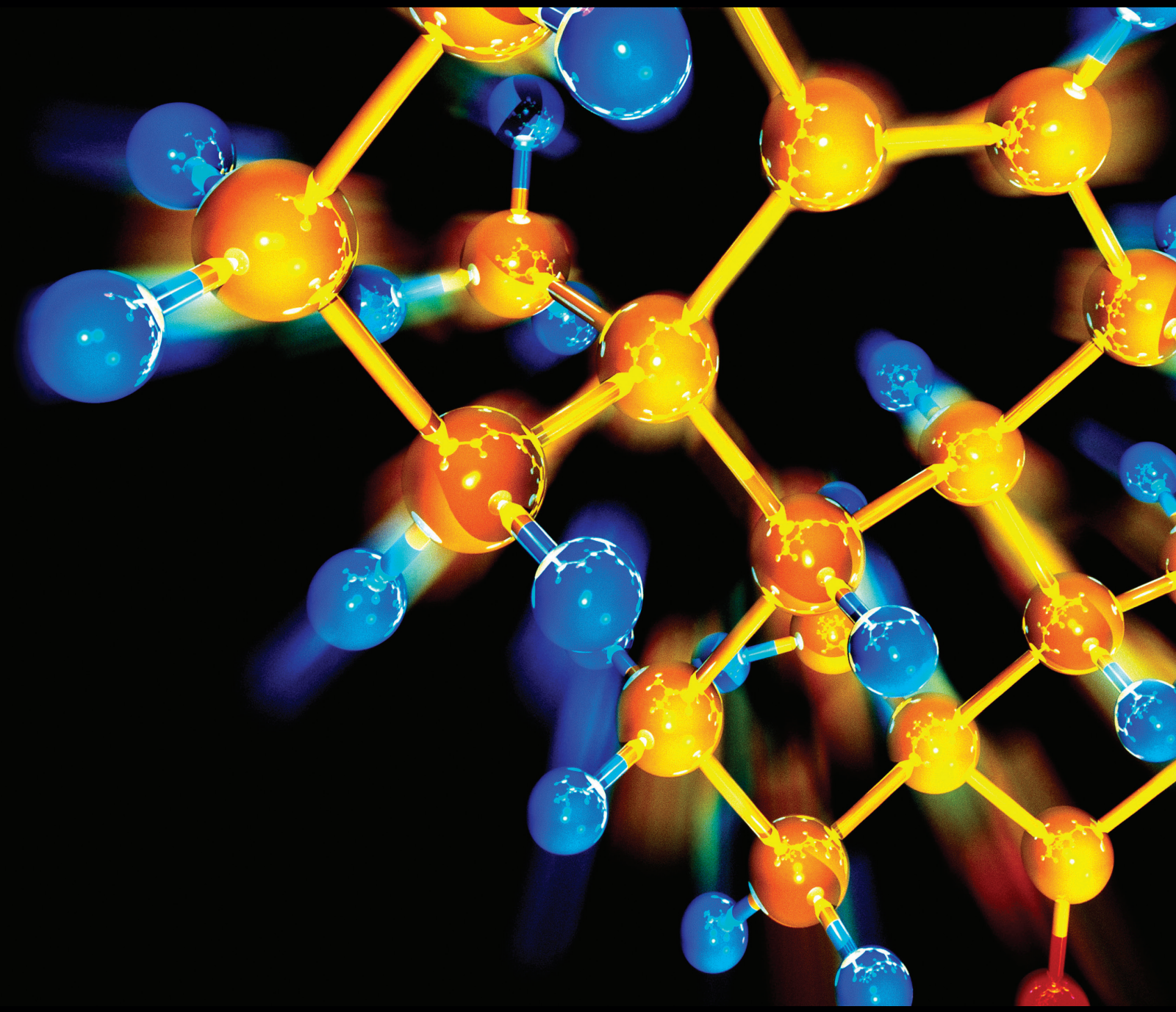
Journal of Chemistry

Separation of Organic and Inorganic Compounds for Specific Applications

Guest Editors: Hasan Uslu, Dragomir Yankov, Kailas L. Wasewar, Saeid Azizian, Najeeb Ullah, and Waqar Ahmad



Separation of Organic and Inorganic Compounds for Specific Applications

Journal of Chemistry

Separation of Organic and Inorganic Compounds for Specific Applications

Guest Editors: Hasan Uslu, Dragomir Yankov, Kailas L. Wasewar, Saeid Azizian, Najeeb Ullah, and Waqar Ahmad

Copyright © 2015 Hindawi Publishing Corporation. All rights reserved.

This is a special issue published in "Journal of Chemistry." All articles are open access articles distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Contents

Separation of Organic and Inorganic Compounds for Specific Applications, Hasan Uslu, Dragomir Yankov, Kailas L. Wasewar, Saeid Azizian, Najeeb Ullah, and Waqar Ahmad
Volume 2015, Article ID 698259, 3 pages

Analysis of Antifungal Components in the Galls of *Melaphis chinensis* and Their Effects on Control of Anthracnose Disease of Chinese Cabbage Caused by *Colletotrichum higginsianum*, Ping-Chung Kuo, Ting-Fang Hsieh, Mei-Chi Lin, Bow-Shin Huang, Jenn-Wen Huang, and Hung-Chang Huang
Volume 2015, Article ID 850103, 12 pages

Chromatographic Methods in the Separation of Long-Chain Mono- and Polyunsaturated Fatty Acids, MaThlgorzata DoThlowy and Alina Pyka
Volume 2015, Article ID 120830, 20 pages

Status of the Reactive Extraction as a Method of Separation, Dipaloy Datta, Sushil Kumar, and Hasan Uslu
Volume 2015, Article ID 853789, 16 pages

Experimental and Modeling Studies on the Prediction of Gas Hydrate Formation, Jian-Yi Liu, Jing Zhang, Yan-Li Liu, Xiao-Hua Tan, and Jie Zhang
Volume 2015, Article ID 198176, 5 pages

Layer-by-Layer Assembly of Polysaccharides and 6,10-Ionene for Separation of Nitrogen-Containing Pharmaceuticals and Their Enantiorecognition by Capillary Electrophoresis, Anna Ioutsi, Elena Shapovalova, Aleksandra Prokhorova, and Oleg Shpigun
Volume 2015, Article ID 836076, 9 pages

Purification of Anthocyanins with o-Dihydroxy Arrangement by Sorption in Cationic Resins Charged with Fe(III), Araceli Castañeda-Ovando, Carlos Andrés Galán-Vidal, Elizabeth Contreras-López, and Ma. Elena Páez-Hernández
Volume 2014, Article ID 367236, 9 pages

Synthesis and Preliminary Properties of Novel Poly(aryl ether)s Containing β-Naphthalene Pendant Group, L. Wang, D. Tao, X. Z. Xiang, and G. M. Zhu
Volume 2014, Article ID 353540, 6 pages

Hindawi Publishing Corporation
Journal of Chemistry
Volume 2015, Article ID 698259, 3 pages
http://dx.doi.org/10.1155/2015/698259

Editorial

Separation of Organic and Inorganic Compounds for Specific Applications

Hasan Uslu,[1] Dragomir Yankov,[2] Kailas L. Wasewar,[3] Saeid Azizian,[4] Najeeb Ullah,[5] and Waqar Ahmad[6]

[1]*Department of Chemical Engineering, Beykent University, 34098 Istanbul, Turkey*
[2]*Institute of Chemical Engineering, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria*
[3]*Advanced Separation and Analytical Laboratory (ASPAL), Department of Chemical Engineering, Visvesvaraya National Institute of Technology, Nagpur 440010, India*
[4]*Department of Physical Chemistry, Faculty of Chemistry, Bu-Ali Sina University, Mahdieh Street, Hamedan 65167, Iran*
[5]*Faculty of Agriculture and Environment, The University of Sydney, Sydney, NSW 2006, Australia*
[6]*Department of Environmental Sciences, Faculty of Agriculture and Environment, The University of Sydney, Room 126, Biomedical Building C81-ATP, Sydney, NSW 2006, Australia*

Correspondence should be addressed to Hasan Uslu; hasanuslu@gmail.com

Received 30 November 2014; Accepted 30 November 2014

Copyright © 2015 Hasan Uslu et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Organic and inorganic compounds deal with the structure, properties, and reactions of compounds. Chemists in general and organic chemists in particular can create new molecules never before proposed which, if carefully designed, may have important properties for the betterment of the human experience. Organic and inorganic compounds play important role in industries such as the rubber, plastics, fuel, pharmaceutical, cosmetics, detergent, coatings, dyestuffs, and agrichemicals industries. The foundations of biochemistry, biotechnology, and medicine are built on organic compounds and their role in life processes. Most of the modern, high-technology materials are composed, at least in part, of organic and inorganic compounds. Clearly, separation of organic and inorganic compounds is critically important to our high standard of living. In this respect, we decided to publish this special issue. We have received many article submissions for this special issue. We selected the best 7 of them for publication. This special issue contains 5 original research papers and 2 review articles.

A study by J.-Y. Liu et al. entitled "Experimental and Modeling Studies on the Prediction of Gas Hydrate Formation" proposed a new prediction model of gas hydrate formation on the base of some kinetics model analysis and kinetic observation of hydrate formation process. The analysis of the present model shows that the formation of gas hydrate is not only relevant to gas composition and free water content but also relevant to temperature and pressure. Through contrast experiment, the predicted result of the new prediction method of gas hydrate crystallization kinetics is close to measured result; it means that the prediction method can reflect the hydrate crystallization accurately. In actual application, the various parameters should be modified according to the practical situation.

In another study by L. Wang et al. entitled "Synthesis and Preliminary Properties of Novel Poly(aryl ether)s Containing β-Naphthalene Pendant Group," the authors synthesized two novel poly(aryl ether)s containing β-naphthalene pendant group and the structures of these polymers were determined by using HNMR spectroscopy. These polymers exhibited good thermal stabilities with high Tg of 256°C and 274°C, respectively. They investigated solubility of polymers in common organic solvents, such as DMAc, DMSO, CH_2Cl_2, and $CHCl_3$ by electrospinning into microfiber (1–5 μm) with lots of nanopores (<100 nm) from $CHCl_3$ solution. These fibers showed high hydrophobicity, and the contact angle of fibers is above 120°. As a result of this study, these polymers

had excellent thermal stabilities and good solubility. The fibers with many nanopores were produced by electrospinning, exhibiting high hydrophobicity. The polymers could be potentially used as high temperature materials, waterproof materials, and transport carriers.

Another valuable article entitled "Purification of Anthocyanins with *o*-Dihydroxy Arrangement by Sorption in Cationic Resins Charged with Fe(III)" was published by A. Castañeda-Ovando et al. in this special issue. The authors proposed a new purification method of anthocyanins with *o*-dihydroxy arrangement. This method is based on a ligand-exchange mechanism, using a cationic exchange resin loaded with metallic ions in order to increase the affinity of the resin to the anthocyanin(s) with *o*-dihydroxy arrangement. They used this method to purify the main anthocyanin (cyanidin-3-glucoside; Cy-3-glc) from the anthocyanic methanolic extract of blue corn. The best sorption result was using Fe(III) in its ion form. The purification procedure begins with the formation of a metal-anthocyanin complex (Cy-3-glc-Fe) which was optimal at pH 5, followed by NaOH 0.1 M elution process in order to eliminate anthocyanins without *o*-dihydroxy arrangement, sugars, and organic acids. Finally, the pure anthocyanin is obtained by adding HCl 0.1 M which breaks the metal-anthocyanin complex. The authors proposed Cy-3-glucoside purification method by means of sorption in cationic exchange resin loaded with Fe3+. This is shown to be quite viable for *o*-dihydroxy anthocyanins, because (i) it decreases procedure costs, since it is possible to be carried out in less time unlike the chromatographic methods proposed previously, (ii) the percentage of anthocyanin extraction reached is above 99.99%, and (iii) it is possible to collect the main anthocyanin with high purity and analyze it by other spectroscopic techniques.

The paper entitled "Layer-by-Layer Assembly of Polysaccharides and 6,10-Ionene for Separation of Nitrogen-Containing Pharmaceuticals and Their Enantiorecognition by Capillary Electrophoresis" by A. Ioutsi et al. investigated preparation of two silica capillaries modified layer by layer with 6,10-ionene and N-(3-sulfo, 3-carboxy)-propionyl chitosan (SCPC) and with 6,10-ionene and dextran sulfate (DS). Dynamic coating of the capillary efficiently reduces the adsorption of the background electrolyte, sample matrix components, and analytes on its inner wall. Such coatings effect good reproducibility and sensitivity of determination. Authors demonstrate that separation of beta blockers, calcium channel blockers, alpha-adrenergic agonists, H1-blockers, and diuretics was most efficient and rapid with a capillary modified with dextran sulfate. Tetrahydrozoline, carbinoxamine, and furacilin, which are commonly employed as treatments for allergic rhinitis, were identified in human urea. Their concentrations, independently verified by HPLC, were found to be 5.3 ± 0.8, 6.6 ± 0.5, and $0.9 \pm 0.2\,\mu g\,mL^{-1}$; LOD = 0.07, 0.03, and $0.10\,\mu g\,mL^{-1}$ and LOQ = 1.0, 0.8, and $0.6\,\mu g\,mL^{-1}$, respectively. As a result of this study, the authors reported the optimal conditions for identification and separation of tetrahydrozoline (alpha-adrenergic agonist), carbinoxamine (H1-blocker), and furacilin in human urea. The DS-modified capillaries are also suitable for separation of other nitrogen-containing compounds and their enantiomers.

The other contribution to this special issue is entitled "Analysis of Antifungal Components in the Galls of *Melaphis chinensis* and Their Effects on Control of Anthracnose Disease of Chinese Cabbage Caused by *Colletotrichum higginsianum*" by P.-C. Kuo et al. (from Taiwan) and itproposedfungal pathogens which caused various diseases which resulted in heavy yield and quality losses on plants of commercial interests such as fruits, vegetables, and flowers. In their preliminary experimental results, the methanol extracts of four species of medicinal plants, *Melaphis chinensis*, *Eugenia caryophyllata*, *Polygonum cuspidatum*, and *Rheum officinale,* possessed antifungal activity to causal agent of cabbage anthracnose, *Colletotrichum higginsianum*. Thus, it was conducted to identify and quantify the chemical constituents in these herbs and to assess the antifungal effects of these compounds. Among the tests, the indicator compound methyl gallate from *M. chinensis* was the most effective against the conidial germination. In addition, it exhibited significant effects of controlling anthracnose disease of Chinese cabbage caused by *C. higginsianum* PA-01 in growth chamber. These results indicate that *M. chinensis* may be of potential for further development of plant-derived pesticides for control of anthracnose of cabbage and other cruciferous crops. The present investigation results indicate that the methanol extracts of *M. chinensis, E. caryophyllata*, *P. cuspidatum*, and *R. officinale* may be of potential for further development of plant-derived pesticides for control of anthracnose of cabbage and other cruciferous crops. The developed HPLC analytical methods are convenient and feasible tools for species authentication and quality assessment of the herbal raw materials. It is helpful to monitor the contents of active principles in the herbs for developing new botanical pesticides. According to the experimental data in the present study, methyl gallate showed only 1/80 activity of the pesticide azoxystrobin; however, the herbal extracts would be safer and less dangerous to the ecosystem. These traditional Chinese medicines could be studied further for their cytotoxicity and synergistic effects of different combinations. It would be also potential to study the antifungal mechanism in the future.

A review contribution is from M. Dołowy and A. Pyka with the title "Chromatographic Methods in the Separation of Long-Chain Mono- and Polyunsaturated Fatty Acids." This review presents various chromatographic systems, TLC, HPLC, GC, and also SFC, developed for identification and accurate quantification of long-chain mono- and polyunsaturated fatty acids from different samples with emphasis on selected literature which was published during last decade. Almost all the aspects such as preseparation step of fatty acids (to *cis*- and *trans*-), stationary phase, solvent system, and detection mode were discussed. This literature review, which is focused on the application of the chromatographic methods such as TLC, HPLC, GC, and also SFC in analysis of mono- and polyunsaturated fatty acids (MUFA and PUFA) in different matrices, shows that all of the presented chromatographic methods are suitable in preseparation and quantification of these compounds. The choice of the chromatographic systems depends on the type of the sample to be analyzed

and on the aim of chromatographic analysis (problem which should be solved by means of this technique). For instant, in order to obtain complete fractionation of *"trans-/cis-"* fatty acids from different samples, the traditional Ag-TLC or argentation HPLC (Ag-HPLC) is used. The column chromatography such as GC and HPLC may be used in further steps of chromatographic analysis of unsaturated fatty acids (MUFA and PUFA), especially to determine the quantity of separated fatty acids. In the case of geometrical isomers (*cis-* and *trans-*) and determining of the number and position of double bonds present in studied unsaturated fatty acids, Ag-HPLC and various GC systems are popular. Current literature indicates that the innovations which are implemented into chromatographic systems include modified stationary phases, new derivatizing agents for fatty acids, and also developing of novel detection systems allowing sensitive and reproducible fatty acid analysis from complex samples. Moreover, the new chromatographic systems combined with other instrumental methods such as MS, MS/MS, IR, or two-dimensional GC enable analysis of a large number of MUFA and PUFA in lower quantities, which is required, for example, in food analysis. The results presented in this paper confirm that the chromatographic methods are still a powerful tool in analysis of mono- and polyunsaturated fatty acids.

The last contribution is a review article in this special issue; it is from D. Datta et al., and it is entitled "Status of the Reactive Extraction as a Method of Separation." This review paper presents a state-of-the-art review on the reactive extraction of carboxylic acids from fermentation broths. This paper principally focuses on reactive extraction that is found to be a capable option to the proper recovery methods. Mechanisms of related secondary, tertiary, and quaternary amines and their reactions with carboxylic acids (mono-, di-, and tri-) were presented. Summary of the modeling experimental data and their kinetic studies has existed.

We sincerely hope that this kind of annual issue series will have a long-term impact and can gather a community around it in a short time in much the same way a successful annual conference does.

Acknowledgments

Firstly, we express our sincere thanks and gratitude to the Editorial Board of Journal of Chemistry for including our special issue as an annual issue. We would also like to thank contributors to this special issue for their scientifically sound research/review articles. With great pleasure and respect, we extend our thanks to the reviewers for critical assessment of each paper, their constructive criticisms, and timely response that made this special issue possible.

Hasan Uslu
Dragomir Yankov
Kailas L. Wasewar
Saeid Azizian
Najeeb Ullah
Waqar Ahmad

Hindawi Publishing Corporation
Journal of Chemistry
Volume 2015, Article ID 850103, 12 pages
http://dx.doi.org/10.1155/2015/850103

Research Article

Analysis of Antifungal Components in the Galls of *Melaphis chinensis* and Their Effects on Control of Anthracnose Disease of Chinese Cabbage Caused by *Colletotrichum higginsianum*

Ping-Chung Kuo,[1] Ting-Fang Hsieh,[2] Mei-Chi Lin,[1] Bow-Shin Huang,[1] Jenn-Wen Huang,[3] and Hung-Chang Huang[4]

[1]*Department of Biotechnology, National Formosa University, Yunlin 632, Taiwan*
[2]*Floriculture Research Center, Taiwan Agricultural Research Institute, Council of Agriculture, Executive Yuan, Yunlin 646, Taiwan*
[3]*Department of Plant Pathology, National Chung Hsing University, Taichung 402, Taiwan*
[4]*Plant Pathology Division, Taiwan Agricultural Research Institute, Council of Agriculture, Executive Yuan, Wufeng, Taichung 413, Taiwan*

Correspondence should be addressed to Ping-Chung Kuo; pcckuoo@nfu.edu.tw and Ting-Fang Hsieh; tfhsieh@tari.gov.tw

Received 8 July 2014; Revised 4 September 2014; Accepted 15 September 2014

Academic Editor: Hasan Uslu

Copyright © 2015 Ping-Chung Kuo et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Fungal pathogens caused various diseases which resulted in heavy yield and quality losses on plants of commercial interests such as fruits, vegetables, and flowers. In our preliminary experimental results, the methanol extracts of four species of medicinal plants *Melaphis chinensis, Eugenia caryophyllata, Polygonum cuspidatum*, and *Rheum officinale* possessed antifungal activity to causal agent of cabbage anthracnose, *Colletotrichum higginsianum*. Thus it was conducted to identify and quantify the chemical constituents in these herbs and to assess the antifungal effects of these compounds. Among the tested principles, the indicator compound methyl gallate from *M. chinensis* was the most effective one against the conidial germination. In addition, it exhibited significant effects of controlling anthracnose disease of Chinese cabbage caused by *C. higginsianum* PA-01 in growth chamber. These results indicate that *M. chinensis* may be potential for further development of plant-derived pesticides for control of anthracnose of cabbage and other cruciferous crops.

1. Introduction

There are numerous reports indicating that tissues of some plant species contain antifungal substances, including rhizomes of *Curcuma longa* [1], seeds of *Cassia tora* [2], stem/leaves and flowers of *Lavandula stoechas* [3], and others. For example, seeds of mustard (*Brassica juncea* cv. Bau Sin) are rich in glucosinolate and enzymatic hydrolysis of this compound resulted in the release of allyl isothiocyanate that is highly toxic to *Rhizoctonia solani* Kühn AG-4, causal agent of root rot of cabbage [4]. Some plant species with antifungal properties are also used as medicinal plants. For example, galls of *Melaphis chinensis* [5, 6] and leaves of *Aloe vera* [7] contained toxic substances against plant pathogenic fungi. The *n*-hexane fraction of a cinnamon (*Cinnamomum cassia*) extract exhibited significant inhibition on mycelia growth of *R. solani* [8]. Various essential oils also displayed significant antifungal activity, such as those from *Hypericum linarioides* [9], *Pistacia lentiscus* [10], *Metasequoia glyptostroboides* [11], and *Silene armeria* [12]. The essential oils of cinnamon leaves (*Cinnamomum zeylanicum*) and clove buds (*Eugenia caryophyllata*) also showed highly antifungal activity against *Botrytis cinerea* [13]. Chu et al. reported that the aqueous extracts of *Coptis chinensis* (goldthread), *Polygonum cuspidatum* (Japanese knotweed), *Cinnamomum cassia* (cinnamon), *Rheum officinale* (Chinese rhubarb), *Polygonum multiflorum*, and *Eugenia caryophyllata* (clove) showed inhibitory effects to conidial germination of *Oidium murrayae* [14].

Water-soluble extracts of clove completely inhibited the conidial germination and mycelial growth of *C. higginsianum* at the concentration of 1% (w/v). In addition, clove oil and eugenol were equally effective in reducing disease severity of anthracnose caused by this pathogen in greenhouse [15]. Although the antifungal activities of various plants were extensively reported, there were relatively few studies regarding the antifungal principles in the plant extracts.

Colletotrichum species are important fungal pathogens causing anthracnose disease of numerous economically important crops, including legumes, ornamentals, vegetables, and fruit trees [16–22] and thus are responsible for severe yield losses of cabbage crops in commercial fields in Taiwan [23]. Although these diseases could be successfully controlled by the synthetic chemical fungicides, the utilization of synthetic fungicides led to the development of resistance and environment pollution. The biological control of plant diseases which is recognized as use of metabolites from the natural source is an eco-friendly resolution [24]. In our preliminary experimental results (Table 1), forty herbal extracts were examined for their antifungal activity against *C. higginsianum* PA-01. Most of them displayed inhibition of the fungus and among the tested methanol extracts, four species of medicinal plants, *Melaphis chinensis, Eugenia caryophyllata*, *Polygonum cuspidatum*, and *Rheum officinale*, exhibited the inhibitory percentages between 80.64 ± 3.16 and 91.92 ± 3.00% at the tested concentration (1250 μg/mL). The experimental data indicated that these extracts possessed antifungal activity to causal agent of cabbage anthracnose *C. higginsianum*. However, the chemical nature of the antifungal substances in these Chinese herbs remains unknown. Therefore, the objectives of this study were to identify the compounds and their antifungal activity in four species of medicinal plants. In addition, the indicator compounds were used as standards to quantitatively analyze these medicinal plants with the aid of high performance liquid chromatography (HPLC) and the validation examinations were performed to confirm that these methods were precise and reliable for quality evaluation. Thus they could be utilized to control the quality of herbal preparations to ensure their antifungal activities. Moreover, their effects of controlling anthracnose disease of Chinese cabbage caused by *Colletotrichum higginsianum* PA-01 in growth chamber were also examined.

2. Materials and Methods

2.1. General Procedure. All the solvents including the HPLC-grade methanol were purchased from Merck KGaA (Darmstadt, Germany). The chemical structures of the indicator compounds were identified by comparison of their spectroscopic and physical data with those reported in the literature. Their purities were better than 99.0% as determined by HPLC. Plant materials were extracted using a Major Science LM-570R shaking incubator. High performance liquid chromatography (HPLC) was performed on a Shimadzu LC-20ATseries pumping system equipped with a Shimadzu SPD-20AUV-Vis detector, a Gemini 5u C18 column (4.6 mm × 250 mm, 5 μm), and a SIL-10AF autosampling system.

2.2. Fungal Pathogen and Plant Materials. Two isolates (PA-01 and PA-19) of *C. higginsianum* were used in this study. They were isolated from diseased leaves of cabbage (*Brassica rapa* L. Chinese group) grown in Yunlin, Taiwan. The cultures were maintained on potato dextrose agar (PDA, Difco, USA). Dry powders of all the examined medicinal herbs were purchased from herbal stores in Yunlin, Taiwan. All the purchased materials for the experiments were authenticated by Dr. T. F. Hsieh and the voucher specimens (PCKuo_TFHsieh_ 201001-201040) were deposited in the herbarium of Department of Biotechnology, National Formosa University, Yunlin, Taiwan. Seeds of Chinese cabbage (*B. rapa* L.) were put on a number 1 filter paper (9 cm in diameter, Toyo Roshi Co., Japan) moistened in water and kept in a Petri dish at room temperature (22–25°C) for 1 day. The germinated seeds were sown in peat moss in plastic pots, 128 cells/tray, and 1 seed/cell. After one week, individual seedlings were transplanted to plastic pots (18 cm in diameter) filled with Stender peat substrates (Stender AG, Germany), 3 plants/pot, and kept in a greenhouse for four weeks with daily watering.

2.3. Effect of the Methanol Extracts on Conidial Germination of C. higginsianum. The methanol extracts were tested for inhibition of conidial germination of *C. higginsianum* PA-01 according to the method of Lee and Dean [25]. The isolate was grown on oatmeal agar (50 g/L) at 25°C under continuous fluorescent light. Conidia were harvested from 7- to 10-day-old cultures and the solution was filtered to collect conidial suspension. Ten microliters of conidial suspension (~10^5 conidia/mL) was mixed with ten microliters of different extracts. The cultures were placed in a moistened plastic box, incubated at 25°C for 24 h, and examined for germination of conidia under a compound microscope. There were six replicates for each sample (100 conidia/replicate). Sterile distilled water was used as negative control and azoxystrobin was used as positive control. Inhibition rate of conidia for each treatment was calculated by

$$\text{Inhibition (\%)} = \left[1 - \frac{(\text{conidial germinated with tested compound})}{(\text{conidial germinated in control})}\right] \times 100\%. \quad (1)$$

2.4. Extraction and Fractionation of Medicinal Plants. The galls of *M. chinensis* (60.0 g) were extracted with methanol under reflux (0.5 L × 5 × 8 h), and the crude extracts were concentrated *in vacuo* to give a brown syrup (MCR, 50.0 g). The crude extract was partitioned between ethyl acetate and water to afford ethyl acetate solubles (MCRE, 46.0 g) and water extracts (MCRW, 4.0 g), respectively. The buds of *E. caryophyllata* (30.0 g) were extracted with methanol under reflux (0.2 L × 5 × 8 h), and the crude extracts were concentrated to give a brown syrup (EC, 7.0 g). The crude

Table 1: The preliminary antifungal screening of the different herbal extracts on conidial germination of *Colletotrichum higginsianum* PA-01.

Sample	Inhibition percentage (%)[a]	Sample	Inhibition percentage (%)[a]
Anemarrhena asphodeloides	6.55 ± 2.73	*Lithospermum erythrorhizon*	8.15 ± 4.32
Arctium lappa	3.99 ± 3.14	*Lycium barbarum*	4.90 ± 1.72
Cassia angustifolia	—[b]	*Melaphis chinensis*	**89.86 ± 2.00**
Cassia tora	12.67 ± 2.71	*Morus alba*	—
Carthamus tinctorius	—	*Paeonia lactiflora*	4.30 ± 3.06
Cinnamomum cassia	27.30 ± 3.89	*Polygala tenuifolia*	—
Crataegus pinnatifida	5.89 ± 2.32	*Polygonum cuspidatum*	**80.64 ± 3.16**
Cuscuta chinensis	15.18 ± 1.83	*Prunella vulgaris*	36.44 ± 4.63
Epimedium brevicornum	5.89 ± 3.02	*Prunus armeniaca*	2.92 ± 3.66
Equisetum hyemale	10.75 ± 2.86	*Pueraria lobata*	3.99 ± 2.58
Eucommia ulmoides	2.43 ± 1.60	*Rheum officinale*	**91.92 ± 3.00**
Eugenia caryophyllata	**87.37 ± 4.74**	*Salvia miltiorrhiza*	8.81 ± 2.28
Forsythia suspensa	15.42 ± 4.32	*Scrophularia ningpoensis*	9.73 ± 2.64
Gardenia jasminoides	3.54 ± 2.59	*Scutellaria barbata*	7.25 ± 1.05
Gentiana scabra	6.06 ± 1.55	*Smilax glabra*	8.08 ± 3.46
Hedyotis diffusa	0.51 ± 1.94	*Sophora flavescens*	31.47 ± 3.83
Houttuynia cordata	—	*Sophora tonkinensis*	27.10 ± 4.04
Isatis indigotica	4.33 ± 2.10	*Taraxacum mongolicum*	5.37 ± 2.19
Leonurus japonicus	14.85 ± 2.99	*Zingiber officinale*	24.62 ± 4.51
Ligusticum chuanxiong	8.21 ± 3.62	*Ziziphus jujuba*	28.01 ± 4.88

[a]Percentage of inhibition at 1250 μg/mL (800X dilution) concentration. ($n = 6$). [b]No inhibition was found.

extract was partitioned between ethyl acetate and water to afford ethyl acetate solubles (ECE, 5.5 g) and water extracts (ECW, 1.5 g), respectively. The roots of *P. cuspidatum* (100.0 g) were extracted with methanol under reflux (0.3 L × 5 × 8 h), and the crude extracts were concentrated to give a brown syrup (PC, 8.0 g). The crude extract was partitioned between chloroform and water to afford chloroform solubles (PCC, 1.7 g) and water extracts (PCW, 6.3 g), respectively. The roots of *R. officinale* (100.0 g) were extracted with methanol under reflux (0.3 L × 5 × 8 h), and the crude extracts were concentrated to give a brown syrup (RO, 27.0 g). The crude extract was partitioned between chloroform and water to afford chloroform solubles (ROC, 2.8 g) and water extracts (ROW, 24.2 g), respectively.

2.5. Purification and Identification of Indicator Compounds. The methods for purification and identification of indicator compounds in the four medicinal plants were described as follows.

(I) M. chinensis. The ethyl acetate soluble fraction (MCRE, 40.0 g) of the crude extract was applied to a silica gel column and then eluted with chloroform and step gradient of ethyl acetate (10 : 1 to 1 : 1, v/v) to yield 9 fractions. Fraction 3 was subjected to silica gel column chromatography eluted with *n*-hexane and acetone (10 : 1, v/v) to yield methyl gallate (**2**) (10.5 g). Fraction 8 was further resolved on a silica gel column eluted with chloroform and acetone (5 : 1) to give gallic acid (**1**) (2.3 g).

(II) E. caryophyllata. The ethyl acetate soluble fraction (ECE, 5.5 g) of the crude extract was purified with silica gel column chromatography and eluted with *n*-hexane and step gradient of ethyl acetate (20 : 1 to 1 : 1, v/v) to yield 5 fractions. Fraction 2 was further purified on a silica gel column eluted with chloroform and ethyl acetate (20 : 1, v/v) to give eugenol (**3**) (250.0 mg).

(III) P. cuspidatum and R. officinale. For *P. cuspidatum*, the chloroform soluble fraction (PCC, 1.7 g) of the crude extract was purified with silica gel column chromatography and eluted with chloroform and step gradient of ethyl acetate (50 : 1 to 1 : 1, v/v) to yield 9 fractions. Fraction 2 was further recrystallized with chloroform and ethyl acetate to afford physcion (**6**) (25.0 mg). Fraction 6 was further resolved on a silica gel column eluted with chloroform and step gradient of methanol (100 : 1 to 1 : 1, v/v) to yield emodin (**4**) (30.0 mg). For *R. officinale*, the chloroform soluble fraction (ROC, 2.8 g) of the crude extract was purified with silica gel column chromatography and eluted with chloroform and step gradient of methanol (300 : 1 to 1 : 1, v/v) to afford 11 fractions. Fraction 2 was further purified with the assistance of silica gel column chromatography eluted with *n*-hexane and acetone (100 : 1, v/v) to give chrysophanol (**5**) (28.0 mg).

2.6. Chromatography. The six indicator compounds used in chromatographic analysis were gallic acid (**1**) and methyl gallate (**2**) from *M. chinensis*, eugenol (**3**) from *E. caryophyllata*, emodin (**4**) and physcion (**6**) from *P. cuspidatum*, and chrysophanol (**5**) from *R. officinale*. The analytic conditions

for these chemical constituents were determined by HPLC according to the reported methods in the literature [26–28].

2.7. Preparation of Standard Solutions, Calibration Curves, and Validation of the Analytical Methods. The standard solutions and calibration curves for the six indicator compounds were prepared according to the methods reported in the literature [29]. The reproducibility and precision of detection were measured by repeatedly injecting a ready-made sample pool and expressed as the relative standard deviation of the results. To determine the variance of samples within a day, the same samples were tested at different times within the day. The variance between days was determined by assaying the spiked samples over three consecutive days at the same time each day. The limit of detection (LOD) was determined as the lowest detectable concentration with acceptable accuracy and precision and three times above the noise level (S/N $\geq$ 3). The recovery of the indicator compounds was evaluated using three different concentrations covering the linear range of the standard curve and the peak heights were compared to the standard compounds to calculate the recovery data.

2.8. Effect of the Methanol Extracts, Partially Purified Fractions, and Indicator Compounds on Conidial Germination of C. higginsianum. Each of the four methanol extracts, partially purified fractions, and the six indicator compounds from the plant extracts were tested for inhibition of conidial germination of *C. higginsianum*, isolates PA-01 and PA-19, as described previously [25].

2.9. Effect of Gallic Acid and Methyl Gallate on Control of Anthracnose Disease of Chinese Cabbage Caused by C. higginsianum PA-01 in Growth Chamber. To determine the effect of indicator compounds, gallic acid, and methyl gallate, on control of anthracnose disease of Chinese cabbage, each dilution of gallic acid and methyl gallate derived from galls of *M. chinensis* with the concentration of 125, 250, 500, and 1000 μg/mL was sprayed on 3-week-old Chinese cabbage plants until running water one day prior to the inoculation of *C. higginsianum* PA-01. Plants sprayed with sterile distilled water were used as controls. There were three replicates (pots) for each treatment. Conidial suspensions of *C. higginsianum* were inoculated on each plant at 8 mL/plant and 10^5 conidia/mL, using a compressed air-sprayer (SIL-AIR, Werther International, Italy). All pots were placed in moist plastic bags and kept in a growth chamber at 24°C under 12 h diurnal illumination. The plastic bags were removed after one-day incubation and the plants were examined for lesion number and infection area in 3 cm diameter of leaf spot at 5, 7, and 9 days after inoculation.

2.10. Statistical Analysis. Data collected from all the experiments in this study were analyzed for statistical significance using analysis of variance (ANOVA). Means of treatments in each experiment were separated using Duncan's multiple range tests. The analytical results are expressed as mean ± standard deviation (SD). Relative standard deviations (RSDs) were calculated from those values. In addition, the mean values of lesion number and lesion area on infection leaves were analyzed by the least significant difference (LSD) test.

3. Results and Discussion

3.1. Antifungal Activities of the Crude Extracts. The antifungal effects of the forty herbal extracts on conidial germination of *C. higginsianum* PA-01 are displayed in Table 1. Among the examined samples, four species of medicinal plants, including *Melaphis chinensis, Eugenia caryophyllata*, *Polygonum cuspidatum*, and *Rheum officinale*, displayed significant antifungal activity against *C. higginsianum* PA-01. Thus these four extracts were selected as the targets of developing new botanical pesticides. Although the synthetic fungicides successfully controlled the plant diseases sometimes, they also contributed to increasing the population of fungicide-resistant pathogens [30]. Natural plant metabolites are generally considered as safe to the humans and environment since these chemical compounds are easily decomposed in the soil and would not exhibit long-term effects to the environment [31]. Thus more and more reports were focused on the development of new plant-derived pesticide preparations recently [1, 4, 15, 32, 33], but comparatively few studies related to the antifungal principles in the bioactive extracts were completed. These new preparations would be hopeful to reduce the damage caused by traditional synthetic fungicides and in the meanwhile to suppress the disease development effectively. Detailed chemical analysis of the constituents in the plant extracts is helpful to confirm the active compounds and control the quality of the plant-derived pesticide preparations.

3.2. Identification of Indicator Compounds in the Medicinal Plant Extracts. The indicator compounds (Figure 1), including gallic acid (**1**) [34] and methyl gallate (**2**) [35] from the galls of *M. chinensis*, eugenol (**3**) [36] from buds of *E. caryophyllata*, emodin (**4**) [37] and physcion (**6**) [38] from the roots of *P. cuspidatum*, and chrysophanol (**5**) [39] from the roots of *R. officinale*, were purified and characterized by comparison of their spectral and physical data with those reported in the literature. The purity of all the indicator compounds except physcion (**6**) as determined by HPLC was better than 99.0%.

3.3. Optimization of the HPLC Condition and Method Validation. The optimized HPLC analytical conditions for the medicinal plant extracts were designed as displayed in the experimental section. The calibration curve parameters and limits of detection (LOD) for the indicator compounds were displayed in Table 2. The precision of the HPLC method developed was evaluated through the intraday and interday experiments. Among the linear ranges, the RSDs for all the indicator compounds of the intraday and interday precisions were found to be less than 1.62 and 2.61%, respectively (Table 2). The recovery of the indicator compounds was determined by the addition of a sample with known concentration to the standard solution, and the mean recovery rate was found to be in the ranges from 81.12 to 126.33%

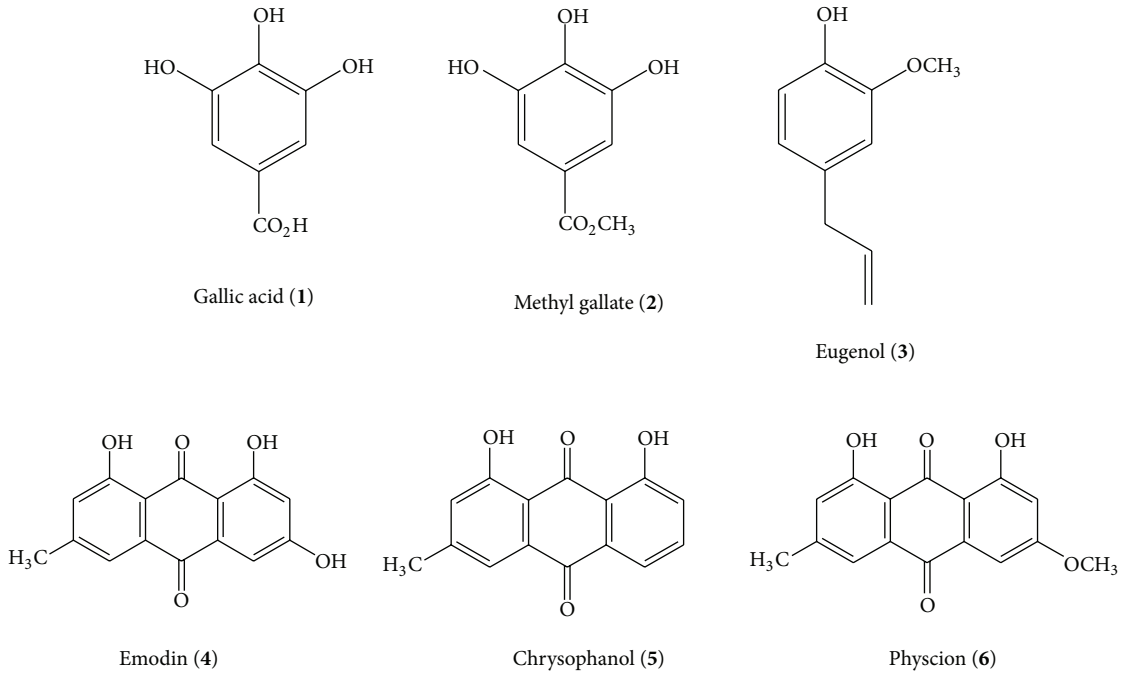


Figure 1: Chemical structures of the indicator compounds **1**–**6**.

with satisfactory RSDs in the ranges between 0.09 and 3.26% (Table 2).

In the present study, the indicator compounds in *M. chinensis*, *E. caryophyllata*, *C. cassia*, *P. cuspidatum*, and *R. officinale* had been extracted and purified. The indicator compounds were further used as standards to quantitatively analyze these traditional Chinese medicines with the aid of HPLC and the validation examinations were carried out to confirm that these methods were precise and reliable for quality evaluation. In the development of the HPLC method for the quantitative determination of indicator compounds, several solvent systems and separation columns were evaluated and compared. Detection wavelength was also optimized in this work. The maximum number and the heights of the peaks of the constituents were obtained and the baseline of chromatogram was stable. The reproducibility of the analytical method was performed and the results showed that it was satisfactory with the RSDs below 3.0% for any of the indicator compounds (data not shown). The precision and recovery tests all displayed that the established HPLC chromatographic methods were valid for the quantitative determination of the indicator compounds and also convenient and feasible as tools for species authentication and quality assessment of the herbal raw materials.

3.4. Quantitative Determination of Indicator Compounds in the Medicinal Plant Extracts. The developed HPLC chromatographic analytical methods were applied to assess the contents of the indicator compounds in the extracts of corresponding plant materials and the data were displayed in Table 3. The contents of methyl gallate (**2**) and eugenol (**3**) in the ethyl acetate soluble fractions of methanol extracts of *M. chinensis* and *E. caryophyllata*, respectively, were more than 30% and they indicated that these constituents were the major component in the plant extracts. In contrast, emodin (**4**), chrysophanol (**5**), and physcion (**6**) were less than 5% in the methanol extracts of *P. cuspidatum* and *R. officinale*. The reproducibility of the analytical results was satisfactory with the RSDs below 3.53% for all the examined indicator compounds.

3.5. Antifungal Activities of the Methanol Extracts, Partially Purified Fractions, and Indicator Compounds. The antifungal effects of the extracts and fractions on conidial germination of *C. higginsianum* PA-01 and PA-19 are displayed in Table 4. Most of the crude extracts and low polarity fractions displayed inhibitions of the conidial germination of the fungal pathogen. Among the tested samples, the ethyl acetate fraction of the methanol extracts of *M. chinensis* exhibited the most significant antifungal activities towards *C. higginsianum* PA-01 and PA-19 with the IC_{50} values of 236.6 and 191.4 μg/mL, respectively. The antifungal effects of the indicator compounds **1**–**6** and the reference compound azoxystrobin were displayed in Table 5. Compounds **1**–**5** all exhibited the inhibitory effects against *C. higginsianum* PA-01 with the IC_{50} values less than 850.3 μg/mL, and comparatively compounds **1**–**4** and **6** showed the significant inhibition of the conidial germination of *C. higginsianum* PA-19 with the IC_{50} values ranging from 22.1 to 1259.0 μg/mL, respectively. The major component methyl gallate (**2**) in the most active fraction (MCRE) displayed the most significant antifungal effects with the IC_{50} values of 40.2

Table 2: Calibration curve parameter, limits of detection (LOD), precision, and recovery for the indicator compounds.

Compound	Calibration curve	Correlation coefficients (r^2)	Linear range (μg/mL)	LOD (μg/mL)	Concentration (μg/mL)	Intraday precision Mean ± SD (RSD %)	Interday precision Mean ± SD (RSD %)	Spiked concentration (μg/mL)	Recovery (%)	RSD (%)
1	$y = 12112x + 33065$	0.9995	2.49–498.00	0.180	9.96	9.02 ± 0.06 (0.63)	8.76 ± 0.23 (2.61)	6.58	118.02 ± 3.85	3.26
					249.00	250.86 ± 0.48 (0.19)	248.87 ± 1.63 (0.65)	13.63	105.45 ± 1.11	1.05
					498.00	497.11 ± 0.67 (0.14)	493.71 ± 2.90 (0.59)	26.67	106.78 ± 1.10	1.10
2	$y = 11797x + 53348$	0.9996	2.50–500.00	0.200	9.96	8.69 ± 0.14 (1.62)	8.57 ± 0.13 (1.25)	26.60	126.33 ± 0.91	0.72
					249.00	252.57 ± 0.71 (0.28)	251.79 ± 1.71 (0.68)	120.33	106.75 ± 0.33	0.31
					498.00	498.75 ± 3.55 (0.71)	498.75 ± 2.51 (0.50)	264.79	105.16 ± 0.43	0.41
3	$y = 12313x + 376$	0.9999	24.50–490.05	0.012	49.01	48.85 ± 0.13 (0.26)	49.61 ± 0.70 (1.42)	102.64	108.61 ± 2.88	2.65
					98.01	98.71 ± 0.36 (0.36)	98.91 ± 0.70 (0.71)	124.62	96.98 ± 0.60	0.62
					490.05	489.95 ± 0.94 (0.19)	498.99 ± 7.68 (1.54)	168.50	93.01 ± 1.20	1.29
4	$y = 83352x - 359078$	0.9998	10.09–50.47	0.010	10.09	10.00 ± 0.02 (0.21)	10.01 ± 0.02 (0.19)	10.09	95.59 ± 0.42	0.44
					25.24	25.40 ± 0.19 (0.73)	25.81 ± 0.60 (2.34)	25.24	89.26 ± 0.13	0.14
					50.47	50.44 ± 0.33 (0.65)	50.31 ± 0.31 (0.61)	50.47	90.41 ± 0.08	0.09
5	$y = 92840x - 376375$	0.9992	9.93–49.66	0.010	9.93	10.33 ± 0.08 (0.82)	10.31 ± 0.05 (0.45)	9.93	81.12 ± 2.15	2.65
					24.83	24.19 ± 0.14 (0.56)	24.17 ± 0.12 (0.52)	24.83	99.81 ± 0.35	0.36
					49.66	49.89 ± 0.10 (0.19)	50.29 ± 0.65 (1.30)	49.66	108.67 ± 0.49	0.45
6	$y = 54687x - 123241$	0.9968	9.75–48.76	0.010	9.75	10.55 ± 0.09 (0.86)	10.61 ± 0.08 (0.72)	9.75	88.64 ± 0.63	0.71
					24.38	23.11 ± 0.06 (0.26)	23.35 ± 0.19 (0.81)	24.83	82.00 ± 0.36	0.44
					48.76	49.24 ± 0.25 (0.50)	49.76 ± 0.51 (1.03)	48.76	94.96 ± 0.74	0.78

Table 3: Contents of each indicator compound in the crudes and fractions of the examined herb extracts.

Compound	Samples											
	MCR	MCRE	MCRW	EC	ECE	ECW	PC	PCC	PCW	RO	ROC	ROW
1	4.84 ± 0.14 (2.90)[a]	5.20 ± 0.17 (3.35)	4.66 ± 0.12 (2.63)	—	—	—	—	—	—	—	—	—
2	29.57 ± 0.32 (1.08)	36.24 ± 1.28 (3.53)	6.02 ± 0.15 (2.47)	—	—	—	—	—	—	—	—	—
3	—[b]	—	—	26.12 ± 0.70 (2.68)	43.83 ± 1.15 (2.63)	N.D.[c]	—	—	—	—	—	—
4	—	—	—	—	—	—	0.46 ± 0.01 (1.08)	2.93 ± 0.01 (0.28)	N.D.	2.36 ± 0.00 (0.13)	2.15 ± 0.01 (0.65)	N.D.
5	—	—	—	—	—	—	N.D.	N.D.	N.D.	3.71 ± 0.01 (0.30)	2.06 ± 0.00 (0.09)	N.D.
6	—	—	—	—	—	—	0.59 ± 0.01 (1.30)	3.94 ± 0.01 (0.32)	N.D.	1.38 ± 0.01 (0.82)	1.79 ± 0.01 (0.55)	N.D.

[a]The contents of each compound were presented as mean ± S.D. (RSD %) (%, g/g sample). [b]Not determined. [c]Not detectable.

Table 4: Antifungal activity of crude extracts and fractions of Chinese medicinal herbs[a].

Sample	*C. higginsianum* PA-01			*C. higginsianum* PA-19		
	Concentration (μg/mL)	Inhibition of conidial germination (%)[b]	IC_{50} (μg/mL)	Concentration (μg/mL)	Inhibition of conidial germination (%)	IC_{50} (μg/mL)
MCR	315.63	16.27 ± 4.88***	699.7	303.13	6.35 ± 2.95***	673.5
	631.25	59.35 ± 3.67***		606.25	49.92 ± 5.87***	
	1262.50	89.86 ± 2.00***		1212.50	97.32 ± 2.17***	
MCRE	76.56	8.02 ± 3.74***	236.6	76.17	18.15 ± 4.70***	191.4
	153.13	24.83 ± 3.76***		152.34	43.94 ± 3.30***	
	306.25	68.64 ± 2.76***		304.69	75.68 ± 3.95***	
MCRW	317.19	5.08 ± 2.63***	918.3	304.69	−0.17 ± 0.41***	984.4
	634.38	20.07 ± 4.73***		609.38	4.87 ± 4.21***	
	1268.75	77.15 ± 5.99***		1218.75	81.74 ± 4.04	
EC	318.75	16.47 ± 6.76***	671.3	310.94	23.74 ± 4.08***	622.1
	637.50	60.45 ± 5.66***		621.88	47.06 ± 3.97***	
	1275.00	87.37 ± 4.74***		1243.75	94.07 ± 3.11***	
ECE	314.06	43.46 ± 5.34***	381.0	303.13	44.78 ± 5.10***	397.8
	628.13	69.52 ± 6.19***		606.25	61.85 ± 4.20***	
	1256.25	94.54 ± 3.47***		1212.50	99.15 ± 1.00***	
ECW	340.63	7.81 ± 3.27***	884.4	304.69	6.73 ± 5.18**	557.4
	681.25	43.32 ± 8.70***		609.38	40.67 ± 6.45***	
	1362.50	82.76 ± 4.27***		1218.75	76.10 ± 4.16***	
PC	314.06	0.51 ± 1.90***	839.6	300.00	0.83 ± 1.10	864.0
	628.13	22.97 ± 4.53***		600.00	28.76 ± 4.58***	
	1256.25	91.92 ± 3.00***		1200.00	85.95 ± 3.59***	
PCC	326.56	14.60 ± 3.31***	718.1	300.00	1.34 ± 1.38	757.2
	653.13	54.05 ± 5.72***		600.00	49.33 ± 5.45***	
	1306.25	98.15 ± 2.34***		1200.00	88.29 ± 2.17***	
PCW	326.56	0.68 ± 3.07***	—[c]	326.56	0.50 ± 1.21	—[c]
	653.13	5.74 ± 4.05***		653.13	6.69 ± 2.62	
	1306.25	42.09 ± 5.52***		1306.25	12.54 ± 3.56	
RO	331.25	5.09 ± 4.71***	925.6	312.50	1.00 ± 1.17	843.6
	662.50	40.88 ± 4.78***		625.00	32.27 ± 3.95***	
	1325.00	80.64 ± 3.16***		1250.00	86.12 ± 3.72***	
ROC	318.75	19.69 ± 4.34***	680.9	309.38	1.34 ± 1.05	736.6
	637.50	43.41 ± 4.27***		618.75	49.50 ± 4.43***	
	1275.00	91.75 ± 3.75***		1237.50	99.50 ± 0.84***	
ROW	326.56	0.34 ± 2.76***	884.1	306.25	0.50 ± 1.21	984.5
	653.13	37.16 ± 5.84***		612.50	17.06 ± 3.40***	
	1306.25	79.29 ± 3.60***		1225.00	75.59 ± 3.95***	

[a] $n = 6$; [b] mean ± S.D.; ** $P < 0.01$; *** $P < 0.001$; [c] $IC_{50} > 1306.25$ μg/mL and not determined.

and 22.1 μg/mL towards *C. higginsianum* PA-01 and PA-19 (Figure 2), respectively, compared to the reference synthetic pesticide azoxystrobin (IC_{50} 0.5 and 0.4 μg/mL against *C. higginsianum* PA-01 and PA-19, resp.). Among the examined samples, most of them displayed significant inhibition of the conidial germination of the pathogen and this indicated that these plant-derived pesticide preparations were promising.

3.6. Effect of Gallic Acid and Methyl Gallate on Control of Anthracnose Disease of Chinese Cabbage Caused by C. higginsianum PA-01 in Growth Chamber. Suppression of Chinese cabbage anthracnose by indicator compounds, gallic acid, and methyl gallate was dependent on the concentration, where lesion area (%) and lesion number per 3 cm in diameter of infected leaf were decreased by increasing concentrations of each indicator compound (Table 6). In general, disease

Table 5: Antifungal activity of indicator compounds[a].

Compound	*C. higginsianum* PA-01			*C. higginsianum* PA-19		
	Concentration (μg/mL)	Inhibition of conidial germination (%)[b]	IC_{50} (μg/mL)	Concentration (μg/mL)	Inhibition of conidial germination (%)	IC_{50} (μg/mL)
1	323.44	20.00 ± 3.19***	586.4	307.81	34.45 ± 5.73***	361.6
	646.88	76.19 ± 4.36***		615.63	81.34 ± 3.42***	
	1293.75	98.28 ± 1.68***		1231.25	99.50 ± 0.84***	
2	19.73	25.81 ± 5.67***	40.2	9.42	25.96 ± 3.63***	22.1
	39.45	64.02 ± 4.34***		18.85	47.56 ± 3.61***	
	78.91	84.98 ± 4.03***		37.70	76.05 ± 2.50***	
3	318.75	13.93 ± 3.23***	716.5	320.31	34.01 ± 4.12***	434.5
	637.50	53.13 ± 4.37***		640.63	79.16 ± 3.97***	
	1275.00	84.14 ± 3.96***		1281.25	99.32 ± 1.23***	
4	310.94	10.40 ± 2.47***	749.1	325.00	2.67 ± 2.41	759.9
	621.88	43.82 ± 4.53***		650.00	60.20 ± 2.95***	
	1243.75	84.48 ± 3.52***		1300.00	87.12 ± 2.32***	
5	310.94	9.56 ± 3.68***	850.3	300.00	0.83 ± 0.63	—[d]
	621.88	52.12 ± 5.11***		600.00	1.67 ± 1.27	
	1243.75	73.28 ± 4.31***		1200.00	8.19 ± 1.65	
6	326.56	2.35 ± 3.49***	—[c]	323.44	3.34 ± 2.74	1210.8
	653.13	14.21 ± 5.77***		646.88	23.08 ± 4.00***	
	1306.25	29.83 ± 4.36***		1293.75	50.84 ± 5.78***	
Azoxystrobin	0.57	37.96 ± 2.41***	0.5	0.30	38.99 ± 3.54***	0.4
	1.15	66.78 ± 7.56***		0.60	69.13 ± 3.03***	
	2.29	81.48 ± 2.76***		1.20	88.53 ± 2.61***	

[a]$n = 6$; [b]mean ± S.D.; ***$P < 0.001$; [c]$IC_{50} > 1306.25$ μg/mL and not determined; [d]$IC_{50} > 1200.00$ μg/mL and not determined.

Table 6: Effect of gallic acid and methyl gallate on control of anthracnose disease of Chinese cabbage caused by *Colletotrichum higginsianum* PA-01 in growth chamber.

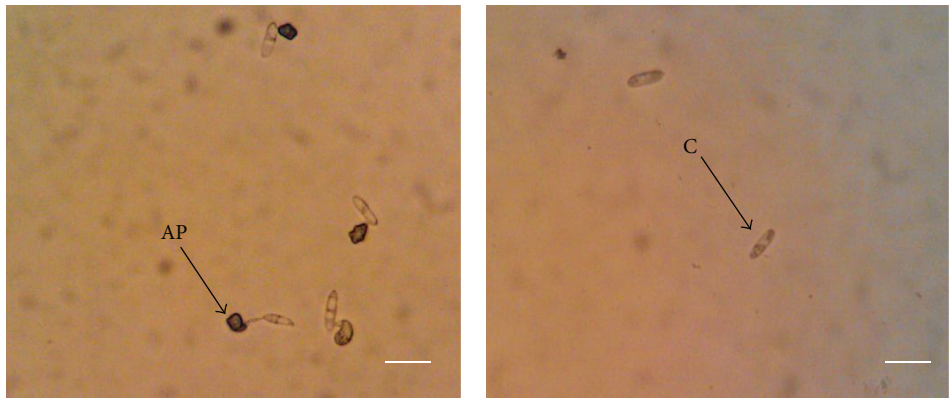
Treatment	Conc. (μg/mL)	5 days[1]		7 days		9 days	
		LN[2]	LA (%)	LN	LA (%)	LN	LA (%)
CK	0	18.3[a3]	40.6[a]	27.3[a]	64.4[a]	34.8[a]	81.6[a]
Gallic acid	125	7.1[b]	16.6[b]	10.7[b]	25.5[b]	23.6[b]	69.1[b]
	250	4.4[cd]	10.6[cd]	9.6[bc]	24.0[bc]	20.1[cd]	56.4[c]
	500	4.0[cd]	10.3[cd]	8.6[bcd]	23.3[bc]	17.8[de]	55.5[c]
	1000	4.0[cd]	8.7[cd]	6.7[cde]	19.7[cd]	13.2[f]	38.4[d]
Methyl gallate	125	4.7[c]	11.6[c]	9.4[bc]	21.0[bcd]	21.3[bc]	54.2[c]
	250	4.6[c]	11.3[cd]	7.7[bcd]	20.3[cd]	19.0[cd]	52.1[c]
	500	3.4[d]	7.9[d]	6.0d[e]	17.9[d]	14.9[ef]	40.9[d]
	1000	1.3[e]	3.6[e]	3.9[e]	9.9[e]	7.2[g]	23.8[e]
$LSD_{0.05}$		1.23	3.55	2.95	4.86	3.36	7.54

[1]Days after inoculation. [2]LN: lesion number per 3 cm in diameter of infected leaves and LA (%): percentage of lesion area per 3 cm in diameter of infected leaves. [3]Data in each column with the same letter are significantly different according to LSD test in $P = 0.05$.

suppression by methyl gallate was better than by gallic acid in the same concentration (Figure 3). For example, the lesion number was not a significant difference in treatment of methyl gallate with 14.9 at 500 μg/mL and in treatment of gallic acid with 13.2 at 1000 μg/mL 9 days after inoculation. Similarity, lesion area (%) was also not a significant difference in treatment of methyl gallate with 40.9 at 500 μg/mL and in treatment of gallic acid with 38.4 at 1000 μg/mL 9 days after inoculation (Table 6). It means that lower concentration of methyl gallate displayed better effects for disease control than higher concentration of gallic acid. The results would be valuable for the discovery of new plant-derived pesticide preparations.

4. Conclusion

The present investigation results indicate that the methanol extracts of *M. chinensis, E. caryophyllata*, *P. cuspidatum*,



(a) (b)

Figure 2: Effect of methyl gallate on inhibition of conidial germination of *Colletotrichum higginsianum* PA-19. (a) An irregular brown appressorium (AP) formed after conidial germination in distilled water (check); and (b) conidium (C) failed to germinate in the solution with 22.1 μg/mL of methyl gallate (bar scale = 20 μm).

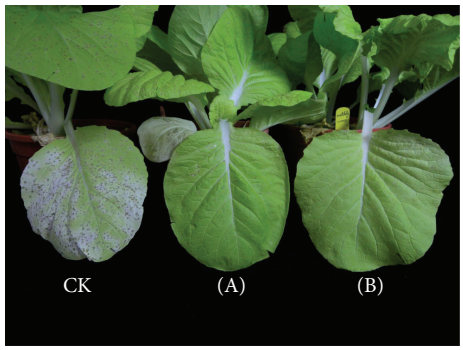


Figure 3: Effect of gallic acid (1000 μg/mL) (A) and methyl gallate (1000 μg/mL) (B) on control of anthracnose disease of Chinese cabbage caused by *Colletotrichum higginsianum* PA-01.

and *R. officinale* may be potential for further development of plant-derived pesticides for control of anthracnose of cabbage and other cruciferous crops. The developed HPLC analytical methods are convenient and feasible tools for species authentication and quality assessment of the herbal raw materials. They are helpful to monitor the contents of active principles in the herbs for developing new botanical pesticides.

According to the experimental data in the present study, methyl gallate showed only 1/80 activity of the pesticide azoxystrobin; however, the herbal extracts would be safer and less dangerous to the ecosystem. These traditional Chinese medicines could be studied further for their cytotoxicity and synergistic effects of different combinations. It would be also potential to study the antifungal mechanism in the future.

Abbreviation List

HPLC: High performance liquid chromatography
PDA: Potato dextrose agar
LOD: Limit of detection
SD: Standard deviation
RSD: Relative standard deviation.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors are grateful for the financial support from the Council of Agriculture, Executive Yuan, Taiwan, awarded to Dr. P. C. Kuo. This study is supported in part by grants awarded to Dr. T. F. Hsieh and Dr. P. C. Kuo from the Ministry of Science and Technology, Taiwan.

References

[1] M. K. Kim, G. J. Choi, and H. S. Lee, "Fungicidal property of *Curcuma longa* L. rhizome-derived curcumin against phytopathogenic fungi in a greenhouse," *Journal of Agricultural and Food Chemistry*, vol. 51, no. 6, pp. 1578–1581, 2003.

[2] Y.-M. Kim, C.-H. Lee, H.-G. Kim, and H.-S. Lee, "Anthraquinones isolated from *Cassia tora* (Leguminosae) seed show an antifungal property against phytopathogenic fungi," *Journal of Agricultural and Food Chemistry*, vol. 52, no. 20, pp. 6096–6100, 2004.

[3] A. Angioni, A. Barra, V. Coroneo, S. Dessi, and P. Cabras, "Chemical composition, seasonal variability, and antifungal activity of *Lavandula stoechas* L. ssp. stoechas essential oils from stem/leaves and Flowers," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 12, pp. 4364–4370, 2006.

[4] W. C. Chung, J. W. Huang, H. C. Huang, and J. F. Jen, "Effect of ground *Brassica* seed meal on control of *Rhizoctonia* damping-off of cabbage," *Canadian Journal of Plant Pathology*, vol. 24, no. 2, pp. 211–218, 2002.

[5] Y.-J. Ahn, H.-S. Lee, H.-S. Oh, H.-T. Kim, and Y.-H. Lee, "Antifungal activity and mode of action of Galla rhois-derived phenolics against phytopathogenic fungi," *Pesticide Biochemistry and Physiology*, vol. 81, no. 2, pp. 105–112, 2005.

[6] W.-C. Ho, H.-J. Su, J.-W. Li, and W.-H. Ko, "Effect of extracts of Chinese medicinal herbs on spore germination of Alternaria brassicicola, and nature of an inhibitory substance from gallnuts of Chinese sumac (*Rhus chinensis*)," *Canadian Journal of Plant Pathology*, vol. 28, no. 4, pp. 519–525, 2006.

[7] D. Jasso De Rodríguez, D. Hernández-Castillo, R. Rodríguez-García, and J. L. Angulo-Sánchez, "Antifungal activity in vitro of *Aloe vera* pulp and liquid fraction against plant pathogenic fungi," *Industrial Crops and Products*, vol. 21, no. 1, pp. 81–87, 2005.

[8] V.-N. Nguyen, D.-M. Nguyen, D.-J. Seo, R.-D. Park, and W.-J. Jung, "Antimycotic activities of cinnamon-derived compounds against *Rhizoctonia solani* in vitro," *BioControl*, vol. 54, no. 5, pp. 697–707, 2009.

[9] A. Cakir, S. Kordali, H. Kilic, and E. Kaya, "Antifungal properties of essential oil and crude extracts of *Hypericum linarioides* Bosse," *Biochemical Systematics and Ecology*, vol. 33, no. 3, pp. 245–256, 2005.

[10] A. Barra, V. Coroneo, S. Dessi, P. Cabras, and A. Angioni, "Characterization of the volatile constituents in the essential oil of *Pistacia lentiscus* L. from different origins and its antifungal and antioxidant activity," *Journal of Agricultural and Food Chemistry*, vol. 55, no. 17, pp. 7093–7098, 2007.

[11] V. K. Bajpai, A. Rahman, and S. C. Kang, "Chemical composition and anti-fungal properties of the essential oil and crude extracts of *Metasequoia glyptostroboides* Miki ex Hu," *Industrial Crops and Products*, vol. 26, no. 1, pp. 28–35, 2007.

[12] V. K. Bajpai, S. Shukla, and S. C. Kang, "Chemical composition and antifungal activity of essential oil and various extract of *Silene armeria* L," *Bioresource Technology*, vol. 99, no. 18, pp. 8903–8908, 2008.

[13] C. L. Wilson, J. M. Solar, A. El Ghaouth, and M. E. Wisniewski, "Rapid evaluation of plant extracts and essential oils for antifungal activity against *Botrytis cinerea*," *Plant Disease*, vol. 81, no. 2, pp. 204–210, 1997.

[14] Y. L. Chu, W. C. Ho, and W. H. Ko, "Effect of Chinese herb extracts on spore germination of *Oidium murrayae* and nature of inhibitory substance from Chinese rhubarb," *Plant Disease*, vol. 90, no. 7, pp. 858–861, 2006.

[15] C. L. Lin, T. C. Lin, and J. W. Huang, "Evaluation for efficacy of clove oil and plant nutrients on controlling the cruciferous vegetable anthracnose caused by *Colletotrichum higginsianum*," *Plant Pathology Bulletin*, vol. 19, pp. 167–176, 2010.

[16] J. A. Bailey and M. J. Jeger, *Colletotrichum: Biology, Pathology and Control*, CAB International, Wallingford, UK, 1992.

[17] R. D. Fitzell and C. M. Peak, "The epidemiology of anthracnose disease of mango: inoculum sources, spore production and dispersal," *Annals of Applied Biology*, vol. 104, pp. 53–59, 1984.

[18] S. Freeman, T. Katan, and E. Shabi, "Characterization of *Colletotrichum* species responsible for anthracnose diseases of various fruits," *Plant Disease*, vol. 82, no. 6, pp. 596–605, 1998.

[19] R. Thangavelu, P. Sundararaju, and S. Sathiamoorthy, "Management of anthracnose disease of banana caused by *Colletotrichum musae* using plant extracts," *Journal of Horticultural Science and Biotechnology*, vol. 79, no. 4, pp. 664–668, 2004.

[20] R. O'Connell, C. Herbert, S. Sreenivasaprasad, M. Khatib, M.-T. Esquerré-Tugayé, and B. Dumas, "A novel *Arabidopsis-Colletotrichum* pathosystem for the molecular dissection of plant-fungal interactions," *Molecular Plant-Microbe Interactions*, vol. 17, no. 3, pp. 272–282, 2004.

[21] Y. Narusaka, M. Narusaka, P. Park et al., "RCH1, a locus in Arabidopsis that confers resistance to the hemibiotrophic fungal pathogen *Colletotrichum higginsianum*," *Molecular Plant-Microbe Interactions*, vol. 17, no. 7, pp. 749–762, 2004.

[22] A. Huser, H. Takahara, W. Schmalenbach, and R. O'Connell, "Discovery of pathogenicity genes in the crucifer anthracnose fungus *Colletotrichum higginsianum*, using random insertional mutagenesis," *Molecular Plant-Microbe Interactions*, vol. 22, no. 2, pp. 143–156, 2009.

[23] C. L. Lin and J. W. Huang, "The occurrence of cruciferous vegetable anthracnose in Taiwan and identification of its pathogen," *Plant Pathology Bulletin*, vol. 11, pp. 173–178, 2002.

[24] P.-C. Kuo, T.-C. Lin, C.-W. Yang, C.-L. Lin, G.-F. Chen, and J.-W. Huang, "Bioactive saponin from tea seed pomace with inhibitory effects against *Rhizoctonia solani*," *Journal of Agricultural and Food Chemistry*, vol. 58, no. 15, pp. 8618–8622, 2010.

[25] Y. H. Lee and R. A. Dean, "cAMP regulates infection structure formation in the plant pathogenic fungus *Magnaporthe grisea*," *Plant Cell*, vol. 5, no. 6, pp. 693–700, 1993.

[26] J. J. Lu, Y. Wei, and Q. P. Yuan, "Preparative separation of gallic acid from Chinese traditional medicine by high-speed countercurrent chromatography and followed by preparative liquid chromatography," *Separation and Purification Technology*, vol. 55, no. 1, pp. 40–43, 2007.

[27] Z.-D. He, C.-F. Qiao, Q.-B. Han et al., "Authentication and quantitative analysis on the chemical profile of Cassia Bark (*Cortex cinnamomi*) by high-pressure liquid chromatography," *Journal of Agricultural and Food Chemistry*, vol. 53, no. 7, pp. 2424–2428, 2005.

[28] G. Qian, S.-Y. Leung, L. U. Guanghua, and K. S.-Y. Leung, "Differentiation of Rhizoma et Radix *Polygoni Cuspidati* from closely related herbs by HPLC fingerprinting," *Chemical and Pharmaceutical Bulletin*, vol. 54, no. 8, pp. 1179–1186, 2006.

[29] P. C. Kuo and Y. L. Chang, "Fingerprint chromatogram analysis of methanol extracts of *Gardenia jasminoides* and *Arctium lappa* by high performance liquid chromatography," *Analystical Chemistry*, vol. 11, pp. 162–168, 2012.

[30] K. J. Brent, D. W. Hollomon, and M. W. Shaw, "Predicting the evolution of fungicide resistance," in *Managing Resistance to Agrochemicals*, M. B. Green, H. M. LeBaron, and W. K. Moberg, Eds., pp. 303–319, American Chemical Society, Washington, DC, USA, 1990.

[31] H. C. Huang and C. H. Chou, "Impact of plant disease biocontrol and allelopathy on biodiversity and agricultural sustainability," *Plant Pathology Bulletin*, vol. 14, no. 1, pp. 1–12, 2005.

[32] M. Muto, H. Takahashi, K. Ishihara, H. Yuasa, and J. W. Huang, "Control of black leaf spot (*Alternaria brassicicola*) of crucifers by extracts of black nightshade (*Solanum nigrum*)," *Plant Pathology Bulletin*, vol. 14, pp. 25–36, 2005.

[33] J. H. Yen, D. Y. Chen, W. C. Chung, T. T. Tsay, and T. F. Hsieh, "Effect of natural plant protectants on controlling plant-parasitic nematode disease," *Plant Pathology Bulletin*, vol. 17, pp. 169–176, 2008.

[34] J. T. Li, B. L. Yin, Y. Liu, L. Q. Wang, and Y. G. Chen, "Monoaromatic constituents of *Dendrobium longicornu*," *Chemistry of Natural Compounds*, vol. 45, pp. 234–236, 2009.

[35] S. E. Choi, J. H. Yoon, H. K. Choi, and M. W. Lee, "Phenolic compounds from the root of Phragmites communis," *Chemistry of Natural Compounds*, vol. 45, no. 6, pp. 893–895, 2009.

[36] C. Y. Chen, H. M. Wang, S. H. Chung, W. L. Lo, W. L. Yang, and S. C. Yang, "Chemical constituents from the roots of

Cinnamomum subavenium," *Chemistry of Natural Compounds*, vol. 46, no. 3, pp. 474–477, 2010.

[37] J. Savard and P. Brassard, "Reactions of ketene acetals-14. The use of simple mixed vinylketene acetals in the annulation of quinones," *Tetrahedron*, vol. 40, no. 18, pp. 3455–3464, 1984.

[38] M. R. Meselhy, "Constituents from Moghat, the roots of *Glossostemon bruguieri* (Desf.)," *Molecules*, vol. 8, no. 8, pp. 614–621, 2003.

[39] T. Sassa, "Overproduction of moniliphenone , a benzophenone biosynthetic intermediate of chloromonilicin , by *Monilinia fructicola*and its anthraquinone precursors," *Agricultural and Biological Chemistry*, vol. 55, no. 1, pp. 95–99, 1991.

Hindawi Publishing Corporation
Journal of Chemistry
Volume 2015, Article ID 120830, 20 pages
http://dx.doi.org/10.1155/2015/120830

Review Article

Chromatographic Methods in the Separation of Long-Chain Mono- and Polyunsaturated Fatty Acids

Małgorzata Dołowy and Alina Pyka

Department of Analytical Chemistry, Faculty of Pharmacy, Medical University of Silesia in Katowice, 4 Jagiellońska, 41-200 Sosnowiec, Poland

Correspondence should be addressed to Alina Pyka; apyka@sum.edu.pl

Received 15 July 2014; Accepted 7 October 2014

Academic Editor: Hasan Uslu

Copyright © 2015 M. Dołowy and A. Pyka. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

This review presents various chromatographic systems, TLC, HPLC, GC, and also SFC, developed for identification and accurate quantification of long-chain mono- and polyunsaturated fatty acids from different samples with emphasis on selected literature which was published during last decade. Almost all the aspects such as preseparation step of fatty acids (*cis* and *trans*), stationary phase, solvent system, and detection mode are discussed.

1. Introduction

Long-chain fatty acids (LC-FA) are organic compounds in which the hydrocarbon chain length may vary from 10 to 30 carbons. The hydrocarbon chain can be saturated or unsaturated (contains one or more double bonds). Based on the number of double bonds, unsaturated fatty acids are classified into the following groups [1, 2]:

(i) monounsaturated fatty acids (monoenoic acids, MUFA), containing one double bond, for example, oleic acid,

(ii) polyunsaturated fatty acids (polyenoic acids, PUFA), having two or more double bonds, for example, γ-linolenic acid,

(iii) eicosanoids, which are derived from polyenoic fatty acids, for example, prostaglandins.

Recent literature data indicate that both monounsaturated and polyunsaturated fatty acids are biological important compounds which play a significant role for the living organisms [3–17]. Human feeding studies during the last ten years demonstrate that PUFA as well as MUFA are the main components of cholesterol-lowering diet [4, 7, 9]. Moreover, there has been a much interest in the effect of MUFA and PUFA on immune and inflammatory system [13]. Among various monounsaturated fatty acids, the most popular is oleic acid (C18:1n-9). It is found in plants (e.g., olive oil), animals, and microorganisms. Olive oil consumption has benefit for colon and breast cancer prevention [8]. The current studies show that oleic acid plays important role in prevention of coronary disease (ability to reduce LDL-cholesterol) [7, 9]. Polyunsaturated fatty acids similar to monounsaturated fatty acids are widely distributed in nature [18]. There are three classes of unsaturated fatty acids common in human tissues [5]: the ω-3 (n-3 PUFA), ω-6 (n-6 PUFA), and ω-9 (n-9 PUFA) fatty acids. To the group of discussed unsaturated fatty acids belongs also demospongic acid, a mixture of very long-chain fatty acids, mainly C_{24}–C_{30} with the atypical 5,9-diunsaturation system. It exists in microorganisms, marine invertebrates, and terrestrial plants [19]. The main sources of omega-3 are fishes, some plants, and green algae [20]. Green oleaginous algae are the potential source of the following ω-3 acids: eicosapentaenoic (EPA), docosahexaenoic (DHA), and also arachidonic acid (AA) from ω-6 group [21–23]. Omega-6 PUFA are present in high concentration in grains as well as in many seeds and meats. From this reason we can notice an increase in the human consumption of seafood during several last years. Oleaginous microorganisms, as alternative sources of PUFA to others such as animal oil products, have been widely studied. Marine fungoid protists (*Thraustochytrids*) like *Schizochytrium* have been found to be as

a novel, excellent DHA and EPA ω-3 fatty acids producers [24, 25]. An excellent review paper performed by Nichols demonstrates that many microorganisms including marine bacteria have been considered the major de novo producer of n-3 PUFA [26]. Available literature data suggest that over the past several years extensive research has been made for the production of PUFA by fungi [27, 28]. As it was reported by Arjuna among various microorganisms, an optimal source of omega-6 polyunsaturated fatty acids specifically γ-linolenic acid (GLA) can be certain fungi [27].

It is well known that PUFA can affect many physiological processes including cardiovascular, neurological, and immune functions, as well as cancer. Consumption of oils rich in n-3 LC-PUFA during pregnancy reduces the risk for early premature birth [12]. Studies with nonhuman primates and human newborns indicate that DHA is essential for the normal functional development of the retina and brain, particularly in premature infants [13, 29]. A new paper prepared by Kaczmarski et al. demonstrated the significant role of linoleic acid and also α-linolenic acid in some symptoms of atopic dermatitis [17]. Therefore, it could be noted that PUFA are important nutraceutical and pharmaceutical targets [22].

Until today there is a lack of knowledge about the function of LC-PUFA in mammalian tissues and cells in which they are found. However, it was stated that very long-chain polyunsaturated fatty acids are known to accumulate in two types of major genetic peroxisomal diseases, Zellweger syndrome and X-linked adrenoleukodystrophy (X-ALD), characterized by neurodegenerative phenotypes [30, 31]. The study of Hama and coworkers showed existence of many LC-PUFA types in specimens from Zellweger patients suggesting the possibility of a new biomarker for peroxisomal diseases [31]. Review paper performed by Agbaga and coworkers [32] summarized the current knowledge of VLC-PUFA to their functional role in the retina which are highly enriched in PUFA with special emphasis on the elongases responsible for their synthesis by ELOVL4 protein. Interest in LC-PUFA was rekindled in 1987 after LC-PUFA were initially detected in bovine retinas by Aveldano [33]. Effect of retinal LC-PUFA on rod and cone photoreceptors was also described by Bennett et al. [34]. Another paper prepared by Butovich confirmed that, among different mammalian tissues, meibum is an exceptionally complex mixture of various fatty acids [35].

Because most of LC-PUFA including omega-3 and omega-6 fatty acids cannot be synthesized in enough quantity by the human organism, they must be supplied in the diet. The problem of supplementation of LC-PUFA and the exploration of a new (alternative to oil fish) source of LC-PUFA (e.g., marine microalgae) is widely described for few years in many papers. For this reason, there is a need to find an effective and rapid method for identification and quantification of newly developed long-chain mono- and polyunsaturated fatty acids in plants and seafood. A set of various tools such as chromatographic methods is also needed to complete the full structural characteristic of PUFA in mammalian samples, for example, in human plasma, brain, retina, or meibum. This is important for the purpose of clinical diagnosis.

Hence, this review presents various chromatographic systems, TLC, HPLC, GC, and also SFC, suitable for preseparation and accurate quantification of long-chain mono- and polyunsaturated fatty acids from different samples with emphasis on selected literature which was published during last decade.

2. Thin-Layer Chromatography (TLC) of Long-Chain Mono- and Polyunsaturated Fatty Acids

Saturated and unsaturated long-chain fatty acids (MUFA and PUFA) are basic structural elements of lipids. Therefore, chromatographic determination of fatty acids composition by TLC including LC-MUFA and LC-PUFA content is mandatory for lipids analysis in food, agricultural, and also in biological samples [36–39]. Moreover, the mono- and polyunsaturated fatty acids can be chromatographically determined by TLC in lipids from aquatic organisms (e.g., marine and freshwater fishes, shell fishes, and marine algae) [40].

It is well known that thin-layer chromatography is a classical method of separation, identification, and quantification of fatty acids [41]. The literature survey from the last decade dedicated to lipids analysis indicates that, among different analytical methods, thin-layer chromatography (TLC) and its modern version high performance thin-layer chromatography (HPTLC) are still very important tool in lipids and also in fatty acids analysis. As it was reported by Fuchs et al. [41], there are many advantages which make TLC very competitive with HPLC (high performance liquid chromatography) in the fatty acids field such as simplicity of use, less expensive cost, small consumption of solvents in comparison with HPLC, availability of analysis of several samples in parallel, and possibility of easy visualization of unsaturated fatty acids after TLC fractionation by use of suitable dyes [41]. Sherma's report regarding TLC analysis in food and agriculture samples confirmed that quantitative HPTLC equipped with densitometer can produce results comparable to those obtained by the use of gas chromatography (GC) or high performance liquid chromatography (HPLC) [42].

Modern topic in TLC analysis of mono- and polyunsaturated fatty acids is the use of high performance thin-layer chromatography in combination with mass spectrometric detection, for example, HPTLC-MALDI-TOF/MS [43–48]. Research studies indicate that MS detection is a powerful tool for the identification of TLC spots in more detail in comparison with traditional staining methods [41].

Many types of stationary phases classified as normal (NP) and reversed (RP) are used for TLC analysis of fatty acids (MUFA and PUFA). The most popular NP layers are silica gel, alumina, cellulose, starch, polyamides, and kieselguhr [42]. Of all above-mentioned stationary phases, the best is silica gel, which can be additionally modified by impregnation with different agents. Reversed-phase TLC is usually performed on chemically bonded RP-18, RP-2, or RP-8 layers [42].

Numerous research papers by Nikolova-Damyanova and other authors showed that the main reason which explains

why TLC plays a significant role in fatty acid analysis is availability of various commercial and home-made adsorbents including impregnated TLC plates [36, 41, 49–51]. Impregnation of TLC plates with a proper reagent can improve the resolution of different classes of organic compounds including fatty acids. Among different modifications of stationary phases used in TLC, impregnated silica gel is a very suitable adsorbent for MUFA and PUFA analysis [41].

2.1. Separation of MUFA and PUFA with the Use of Impregnated TLC Plates. One of the primarily used TLC impregnating procedure to separate fatty acids in complex lipid samples was silver ion TLC (Ag-TLC). Detailed information on Ag-TLC of saturated and unsaturated fatty acids was performed in several papers and books [36, 49–51]. Silver ion chromatography is based on the ability of Ag^+ to form weak reversible charge transfer complexes with π electrons of the double bonds of unsaturated fatty acids [36, 52]. The retention of long-chain unsaturated fatty acids depends on the strength of complexation with Ag (I), on the number of double bonds and their configuration, and also on the distance between double bonds. Literature data indicate that both home-made and precoated glass plates are used in Ag-TLC [51]. General procedures of preparation of the stationary phases for silver ion chromatographic techniques have been surveyed by Momchilova and Nikolova-Damyanova, in 2003 [53]. Among various available TLC adsorbents, silica gel is the main supporting material [53]. Impregnation of thin layer is performed by spraying or immersing the plate in solution of silver nitrate at concentration of 0.5–20% (in the case of immersion), while for the spraying procedure 10–40% solution of silver nitrate is recommended [51, 53]. The mobile phase used in argentation TLC usually consists of two or three components, for example, hexane, petroleum ether, benzene, and toluene. Moreover, small amount of acetone, diethyl ether, ethanol, methanol, or acetic acid may be added to these mobile phases [40]. Of various visualizing agents of spots, those which are most popular for Ag-TLC of fatty acids are 50% ethanol solution of sulfuric acid, phosphomolybdic acid, and a mixture of copper-acetate-phosphoric acid. Another visualizing method is spraying the plates with fluorescent indicator by 2′,7′-dichlorofluorescein in ethanol and next viewing the spots under UV light [36]. General migration rules in Ag-TLC analysis of fatty acids were described by Nikolova-Damyanova and coworkers [53, 54]. According to Nikolova-Damyanova suggestions the retention of fatty acids with more than one double bond depends on the distance between the bonds and the elution order is as follows: separated double bonds fatty acids > interrupted double bonds > conjugated double bonds; longer chain unsaturated fatty acids (LC-PUFA) are held less strongly than shorter chain fatty acids and fatty acids with *trans* double bonds are held less strongly than fatty acids with *cis* double bonds [53, 54].

One of the first Ag-TLC analyses of fatty acids was performed by Wilson and Sargent in 1992 to separate PUFA having physiological interest. PUFA's methyl esters were separated on silica gel 60 TLC plates impregnated with $AgNO_3$. The plates were developed with toluene-acetonitrile (97 : 3, v/v). Visualization of the spots was made by the use of 3% copper acetate-8% orthophosphoric acid. It was stated that this technique is particularly useful for metabolic studies of the chain elongated PUFA [55]. In another work Wilson and Sargent showed that silver nitrate-impregnated TLC plates were helpful in separation of monounsaturated fatty acids (as methyl esters) from polyunsaturated and also from saturated fatty acids, respectively, in metabolic studies of fatty acids by human skin fibroblasts [56]. Next work by Lin et al. indicated that silica gel and hexane-chloroform-diethyl ether-acetic acid (80 : 10 : 10 : 1, v/v/v/v) were good in analysis of docosahexaenoic acid (22 : 6 DHA) in the spermatozoa of monkeys [57]. Individual phospholipids from this sample were separated by another system such as chloroform-methanol-petroleum ether-acetic acid-boric acid (40 : 20 : 30 : 10 : 1.8, v/v/v/v/v). Other literature data confirmed that argentate silica gel chromatography enabled obtaining the high purity eicosapentaenoic acid extracted from microalgae and fish oils [58]. The recent literature reviews which were focused on TLC chromatography show that, among different chromatographic materials, Ag-TCM-TLC (silver-thiolate silica gel) is very stable (in comparison with highly light sensitive Ag-TLC plates) for TLC analysis of unsaturated organic compounds including MUFA and PUFA. Dillon et al. [59] confirmed that Ag-TCM-TLC system operates similar to Ag-TLC by separating fatty acids on the degree of unsaturation (number of double bonds). The results of this analysis are comparable to those obtained by Ag-TLC. Ag-TCM-TLC method was used to analyze some polyhydrocarbons and also methyl esters of unsaturated fatty acids containing from 0 to 6 double bonds in form of methyl esters. A mixture consisting of hexane-ethyl acetate (9 : 1, v/v) was used as mobile phase. Under these conditions complete separation of fatty acids with 0–5 double bonds was observed. Resolution of fatty acids consisting of 6 double bonds from others was not achieved in this case [59].

The results presented in this section show that various commercial silica gel plates were used in separation of unsaturated fatty acids, but some of them are not suitable for derivatization process by methylation of fatty acids on TLC plates and next their quantification by gas chromatography, because it causes the loss of separated fatty acids [60]. Methylation procedure of fatty acids after their previous fractionation on argentate silica gel was used in the analyses of unsaturated fatty acids from lipid-rich seeds. In this case a mixture of hexane-diethyl ether-acetic acid and 70 : 30 : 1 (v/v/v) was used as a mobile phase. The plates were sprayed with 2′,7′-dichlorofluorescein ethanolic solution and next identified under UV lamp (at 365 nm) [60]. The impact of Ag-silica gel on TLC analysis of methylated fatty acids, for example, c9,t11-CLA (isomer of linoleic acid) in human plasma as a prior step before GC quantification was showed by Shahin et al. [61]. Another paper prepared by Kramer et al. demonstrated that the best technique to analyze the CLA and *trans* 18 : 1 isomers in synthetic and animal products is the combination of gas chromatography with Ag-TLC or with Ag-HPLC [62]. Moreover, usage of Ag-TLC in the separation of isomeric forms of EPA and DHA obtained after chemical isomerization of them (during fish oil deodorization) may be found in a paper by Fournier et al. [63].

A new application of Ag-TLC is bioanalysis. A simple and rapid TLC method for analysis of PUFA levels in human blood was developed by Bailey-Hall et al. [64].

It should be pointed out that, besides the modification of silica gel with Ag^+ ions, the following metal salts, Cu(I), Cu(II), Co(III), and Zn(II), can be used for impregnation of TLC plates [41, 65]. Another type of impregnating agent for TLC analysis of fatty acids (MUFA and PUFA) is boric acid. It was stated that the metabolites of arachidonic acid were satisfactorily separated on silica gel impregnated with boric acid as complexing agent and by mobile phase: hexane-diethyl ether (60 : 40, v/v) [41, 65]. Next modification of stationary phase which has impact on resolution effect of fatty acids and their derivatives such as metabolites (e.g., phospholipids) is EDTA and mobile phase containing chloroform-methanol-acetic acid water in volume composition of 75 : 45 : 3 : 1 [41, 66]. Another work showed that efficient separation of five different phospholipids could be achieved by impregnation of TLC plates with 0.4% ammonium sulfate. A mixture of chloroform-methanol-acetic acid-acetone-water in volume composition of 40 : 25 : 7 : 4 : 2 was suitable for this procedure [67].

Besides the above-presented TLC system in normal phase (NP-TLC), unsaturated fatty acids and their metabolites could be separated on RP-TLC plates. One of the first reports which are focused on RP-TLC analysis of PUFA was made by Beneytout and coworkers in 1992 [68]. Beneytout et al. separated arachidonic acid and its metabolites on reversed-phase layer. The plates were silica gel coated with phenylmethylvinylchlorosilane. A mixture of heptane-methyl formate-diethyl ether-acetic acid (65 : 25 : 10 : 2, v/v/v/v) was applied as mobile phase [68].

2.2. 2D-TLC of MUFA and PUFA. Two dimensional TLC (2D-TLC) is one of the newly developed powerful tools to separate various lipids mixture and fatty acids coming from lipids. It is known that 2D-TLC improved the quality of separation, but it is much more time consuming in comparison with very popular 1D-TLC [41]. Literature review showed that 2D-TLC is rather a method of choice for separation of lipids from cell membrane polyphosphoinositides and also of lipid oxidation products in mixture. This analysis is usually performed on silica gel impregnated with magnesium acetate (7.5%) and by solvent system chloroform-methanol-ammonia (5 : 25 : 5, v/v/v) in the first direction and chloroform-acetone-methanol-acetic acid water (6 : 8 : 2 : 2.1, v/v/v/v) in the second dimension [69].

2.3. Detection of Spots and Quantification Methods of MUFA and PUFA. Detection of fatty acids by TLC method is based on their visualization by binding to a dye. As is was reported in excellent review by Fuchs et al. [41] a lot of visualizing reagents suitable for detection of fatty acids are described in the literature. Among them, the most popular reagents are iodine vapors, 2′,7′-dichlorofluorescein, rhodamine 6G, which produce coloured spots, and also primuline, which gives sensitivities in the nanomole range [41]. In case of PUFA intense darkening is achieved after their separation on $AgNO_3$ impregnated TLC plates (as an effect of reduction of Ag^+ to colloid silver), but this method of detection required the presence of aromatic hydrocarbon as mobile phase component [70]. Other visualizing reagents are as the following: sulfuric acid, potassium dichromate in 40% sulfuric acid, or 3–6% solution of cupric acetate in phosphoric acid. Moreover, detection of different fatty acids is possible by PMA (phosphomolybdic acid) and by sulfuryl chloride vapors [41]. Visualization of fatty acid spots is performed by spraying or dipping the plates in solution of respective visualizing agents. Next, the spots are observed under UV light or identified by densitometry. For more detailed characterization of fatty acids which have been separated by thin-layer chromatography, TLC combined with mass spectrometer (TLC-MS) may be used. In this method the spots are eluted from the chromatographic plates with respective solvents and next obtained fatty acids are analyzed by MS. Applying of TLC coupled with MS enables high resolution of identified peaks. Moreover, there is no need to extract sample from the plates prior to this analysis [41]. A novelty in thin-layer chromatographic instrumentation is a TLC in combination with MALDI MS spectrometer (TLC MALDI) [44, 71, 72]. This technique is rather fast and provides spectra that can be relatively simply analyzed and tolerates high sample contamination [41]. The detection limit of fatty acids determined by TLC MALDI might be less than 1 nanogram [41]. It was stated that TLC MALDI could be satisfactorily applied to very complex lipid mixture (e.g., extracts from stem cells) [47]. For example, by means of combined thin-layer chromatography and MALDI-TOF/MS analyses of the total lipid extract of the hyperthermophilic archaeon *Pyrococcus furiosus* were performed [45]. Next modern trend in analysis of lipid profile is the use of TLC method coupled with FID (flame ionization detector) [73]. Chromarod/Iatroscan TLC-FID was successfully used in the analysis of lipid classes and their constituents of fatty acids extracted from seafood. As it was described in the paper by Sinanoglou et al. [73], Iatroscan is an instrument that combines TLC resolution with capacity of quantification by FID. Efficient TLC-FID separation can be achieved by addition of polar solvent system without changing the stationary phase. However, this apparatus allows analyzing in a short time (2-3 hr) in comparison with GC or HPLC about 30 samples [73].

2.4. TLC Separation of cis and trans Isomers of MUFA and PUFA. Since it was reported that the saturated fatty acids indicate correlation with cardiovascular diseases, unsaturated fatty acids have been recommended for replacement of saturated fatty acids in a diet. For this reason, an increase of interest in unsaturated fatty acids such as n-6 and n-3 fatty acids is observed. It is known that the discussed unsaturated fatty acids form specific geometrical isomers. They can be *trans* or *cis* depending on the orientation of double bond. Of all polyunsaturated fatty acids the *trans* PUFA consisting of C18, C20, and C22 chain lengths are usually part of the human diet. Thus, it is very important to detect and quantify them in food products. One of the most popular method obtaining the mono-, di-, and triunsaturated fatty acids in form of geometrical isomers

(*cis* and *trans*), respectively, is thermal or chemical process [63]. Synthesis of *trans* isomers is usually made by food manufacturing (refining, hydrogenation). For instance, *trans* isomers are formed during deodorization (crucial step of refining) of vegetable or fish oils. As it was reported by Fournier et al. [63], the methodologies regarding accurate separation and quantification of *trans* isomers of mono-, di-, and triunsaturated fatty acids by chromatographic methods were developed in the last decade. Ag-TLC is one of the most powerful chromatographic techniques widely applied to separate *cis* and *trans* isomers of LC-PUFA because it is characterized by simplicity, low cost, and efficiency. Geometrical isomers are separated according to their number of double bonds. The efficacy of Ag-TLC for separation of EPA and DHA isomers was confirmed by Fournier et al., in 2006 [63]. For this purpose TLC plates precoated with silica gel and impregnated by $AgNO_3$ were used. A mixture of toluene-methanol in the volume composition 85 : 15 was applied as a mobile for resolution of EPA mono-, di-, tri-, tetra-, penta-*trans* and DHA hexa-*trans* isomers [63]. In another work, in order to determine the profile of *cis* and *trans* isomers of CLA in liver by the TLC method, a mixture of trichloromethane-n-hexane-glacial ethanoic acid 65 : 35 : 1 (v/v/v) and also silica gel plates were applied. Rhodamine solution was used as a visualizing agent. In further steps, after methylation of separated fatty acids they were quantified by GC method [74].

Next paper indicated that preparative silver nitrate thin-layer chromatography was successfully applied to analyze the *cis,cis*-octadecadienoic acid (18 : 2) in commercial samples of bovine butter fat. The detected by Ag-TLC *cis,cis*-5,9-18 : 2 isomer was found for the first time in butter fat [54]. In another food study, ninety-three commercial samples of Bulgarian butter fats manufactured evenly through the year were subjected to quantitative silver nitrate-TLC of fatty acids components (as isopropyl esters), with particular attention to *trans* monoenoic fatty acid content [75]. All performed results indicate that argentation TLC should be an effective analytical technique in fractionation and identification of geometric isomers of unsaturated fatty acids such as PUFA.

Table 1 shows the most efficient TLC systems used for separation and fractionation of fatty acids from various matrices.

3. High Performance Liquid Chromatography (HPLC)

There are few excellent original papers and reviews until today which are focused on usage of liquid column chromatography for the analysis of fatty acids (saturated and unsaturated) and also their related substances in biological, food, and drugs samples [18, 93–96]. In these papers principles of HPLC including the sample preparation, mobile phases, stationary phases, and detection methods were widely performed. Among the earlier review papers, only three of them survey knowledge of long-chain fatty acids. One of them prepared by Rao et al. [94] showed the LC-PUFA analysis with use of HPLC but since 1974 until 1995. Next paper supported by Rezanka and Votruba [96] demonstrated the use of chromatography including HPLC for analysis of very long-chain fatty acids from 1982 to 2001. The third review prepared by Kolanowski and coworkers [97] described the important instrumental methods such as HPLC and also GC for analysis of omega-3-long-chain polyunsaturated fatty acids but in food only. Lack of the overview about the new achievements in HPLC analysis of mono- and polyunsaturated fatty acids with an emphasis on the analysis of long-chain polyunsaturated fatty acids such as omega-3 and omega-6 causes that there is a necessity to perform a complete literature survey from 2002 to 2013 (last decade) with emphasis on application of HPLC for the analysis of all biological important LC-MUFA and LC-PUFA at the analytical levels in various matrices. This paper highlights the modern achievements of the HPLC including the principles of MUFA and PUFA analysis in different matrices: separation modes and the new detection systems which enable quantification of LC-PUFA at nanogram level. The current knowledge of HPLC analysis of mono- and polyunsaturated fatty acid was described on the basis of the works which were published during the past decade (2002–2013).

The use of HPLC for the separation and quantification of fatty acids increased since 1950, when it was applied for first time by Haward and Martin for analysis of some fatty acids [98]. From this time until today a very quick progress in HPLC analysis of all fatty acids including LC-MUFA and LC-PUFA is observed. It is known that, based on nature of stationary phase (solid or liquid), the high performance liquid chromatography is divided into [18] liquid-liquid chromatography (LLC), adsorption chromatography (LSC), and reversed-phase chromatography (RP), which is a combination of LSC and LLC.

Among above-mentioned high performance liquid chromatographic techniques RP-HPLC plays a key role in LC-PUFA analysis [94]. This technique allows separating those fatty acids which cannot be separated by normal phase HPLC. As it was early reported by Rao et al. [94] the retention of fatty acids in RP-HPLC depends on the polarity of the stationary phase, mobile phase, and chemical structure of examined fatty acids. In general, the retention time is proportional to the chain length and the number of double bonds present in examined fatty acids. Additionally, the influence of geometric isomerization of fatty acids plays a very important role in reversed-phase HPLC analysis. It was reported that the *cis* isomers of the fatty acids are generally eluted before *trans* isomers. Similar effect is observed in case of the differences in number of carbons in studied fatty acids. Those with small amount of carbons (short chain) are eluted first in comparison with long-chain fatty acids [94]. The current literature indicates that the reversed-phase HPLC is one of the popular methods used in the field of fatty acids analysis of MUFA and PUFA because its simplicity, reproducibility, and credibility. The results obtained by HPLC are usually comparable with those determined using GC method. Moreover, the time of analysis is comparable to GC technique [94]. However, development of new detector mode such as flame ionization detector (FID) or electrochemical detector (ED) enhances the applicability of HPLC for MUFA and PUFA analysis [93].

Table 1: Survey of some selected TLC conditions useful for separation and identification of MUFA and PUFA from various samples.

Analyte	Stationary phase	Mobile phase	Remarks	Reference
Extract of lipids coming from aquatic organisms containing PUFA	Plain silica gel plate	Hexane-diethyl ether-acetic acid 95 : 5 : 1 (v/v/v)	The plates were sprayed with sulphuric acid and next analyzed densitometrically at 600 nm	[40]
Human plasma lipids containing linoleic acid	Microscope plates coated with silica gel G	Hexane-diethyl ether-acetic acid 90 : 10 : 1 (v/v/v)	The spots were visualized by solution of 2′,7′-dichlorofluorescein in ethanol or by sulfuric acid, respectively	[61]
Deodorized fish oil containing eicosapentaenoic and docosahexaenoic acid	Argentate silica gel	Toluene-methanol 85 : 15 (v/v)	Ag-TLC method	[63]
Seafood containing saturated and unsaturated fatty acids	Silica gel coated Chromarod/Iatroscan	Hexane-diethyl ether-formic acid 99 : 1 : 0.5 (v/v/v) Chloroform-methanol-water 5 : 4 : 1 (v/v/v)	TLC-FID method (TLC coupled with flame ionization detector)	[73]
Milk sample containing linoleic acid	Argentate silica gel	Hexane-diethyl ether 90 : 10 (v/v)	Ag-TLC in combination with UV detection at 234 nm, the spots were visualized by solution of 2′,7′-dichlorofluorescein in methanol	[76]
Cell lipid extract containing PUFA	Silica gel plates	Chloroform-methanol-acetic acid-water 60 : 50 : 1 : 4 (v/v/v) Heptane-diethyl ether-acetic acid 60 : 40 : 2 (v/v/v)	TLC-densitometry, after developing the plates were impregnated with cupric acetate and phosphoric acid	[77]
Fatty acids isolated from lipid-rich seeds	TLC plates (silica gel 60)	Hexane-diethyl ether-acetic acid 70 : 30 : 1 (v/v/v)	TLC with UV detection at 365 nm, ethanolic solution of 2′,7′-dichlorofluorescein was used to visualize the spots	[78]
Fatty acids separated from oleosomes of beach pea	Silica gel TLC	Hexane-diethyl ether-acetic acid 85 : 15 : 2 (v/v/v)	TLC method, iodine vapors were used to visualize the spots	[79]

3.1. Derivatization Methods of Fatty Acids for HPLC Analysis. The most important problem in HPLC study of LC-MUFA and also LC-PUFA is a need to detect them at lower concentration levels such as nanograms or picograms. Derivatization of fatty acids can improve the importance for HPLC analysis parameters such as sensitivity, precision, selectivity, and also a limit of detection and quantification [18, 95]. Different derivatization methods used for quantitative and qualitative determination of fatty acids were widely described in review paper by Rosenfeld in 2002 [99]. Generally, the kind of derivatizing agent depends on the type of detector applied in PUFA analysis including UV absorption, fluorescence, light scattering, and refractive index detectors [94].

UV-VIS detector is the most popular detection system applied in liquid chromatography because it is sensitive and specific. In 1983 Aveldano and coworkers [100] have applied reversed-phase high pressure liquid chromatography with UV detection on octadecylsilyl column to separate a mixture of underivatized unsaturated and saturated fatty acids and their methyl esters coming from mammalian tissues.

Generally in order to facilitate the detection by UV-VIS absorption the following derivatizing reagents for fatty acids are used [93]: phenacyl bromide (PB), p-bromophenacyl bromide (BPB), p-chlorophenacyl bromide (CPB), p-nitrophenacyl bromide which are used in analysis of fatty acids in oils, standards, and blood in the range from ng to pmol [97]. Naphthyl esters (obtained by 2-naphthyl bromide, p-nitrophenacyl, and p-dichlorophenacyl allow for the detection of picograms fatty acids in standard mixture. 2-Nitrophenylhydrazine (NPH) and 2-bromoacetophenone (or α,p-dibromoacetophenone, BAP) convert the fatty acids into specific derivatives which can be detected in biological samples (serum) with detection limits in fmoles. PNB (p-nitrobenzyl) and p-methylthiobenzyl chloride (MTBC) derivatives of fatty acids with detection limits in pmoles are important in coconut oil analysis. Ideal derivatizing reagent for chiral separation of fatty acids is 3,5-dinitrophenyl isocyanate (DNPI) [96].

Next very popular detection system applied in HPLC is fluorescence detector which has higher sensitivity in comparison with UV-VIS detector. For this reason it could quantify the fatty acids composition at picomole and femtomole level. Several fluorescent reagents are used for the derivatization of fatty acids such as 9-diazomethylanthracene (9-DMA) and 9-anthryldiazomethane (ADAM). Anthryl methyl esters of fatty acids can be easily detected in human plasma and serum in picomoles. Other more sensitive derivatives than ADAM are the esters of pyrenyldiazomethane (PDAM). Coumarins and 9-aminophenanthrene (9-AP) are important in detection of fatty acids in environmental and in biological samples in the range from pmoles to fmoles. Other derivatives like dansyl piperazines and acetamides of fatty acids can be detected in serum below 100 fmol range [93, 94, 97]. Recent study performed by Wang and coworkers in 2013 indicates that using a new fluorescent labeling reagent named 1,3,5,7-tetramethyl-8-butyrethylendiamine-difluoroboradiaza-s-indacene (TMBB-EDAN) a sensitive and rapid HPLC technique for the determination of fatty acids in biosamples (e.g., human serum) was developed. With the proposed procedure the limit of detection of fatty acid derivatives was in the 0.2–0.4 nM range [101].

Another type of fatty acids detection is chemiluminescence. This method is based on the prelabeling of the –COOH groups with a proper chemiluminogenic reagent. In practice luminal and its related compound isoluminal have been widely utilized as chemiluminescence derivatization reagents for fatty acids because of their chemiluminescent properties. Described detection system is efficient for the sensitive detection of fatty acids in human serum and plasma in picomoles [93].

In order to detect the substances with oxidizing or reducing properties including fatty acids an electrochemical detector (ED) may be used. This detection mode enables measure of fatty acids in complex biological samples containing different components at nanogram levels. To obtain the higher sensitivity the following derivatizing reagents for ED can be applied: p-aminophenol; 2,4-dimethoxyaniline; 2-bromo-2′-nitroacetophenone; ferrocene derivatives; 2,4-dinitrophenylhydrazine and 3,5-dinitrobenzoyl chloride. Clinical application of HPLC-ED is showed in the paper by Kotani et al. [80]. In this work determination of plasma fatty acids including PUFA such as arachidonic acid and also linoleic by high performance liquid chromatography coupled with electrochemical detector (HPLC-ED) was performed [80]. This procedure may be found suitable for monitoring plasma fatty acids in diabetic patients.

Among different types of detectors used in HPLC analysis of fatty acids, very useful is that detector which requires none of the preliminary derivatization procedures of studied compounds like, for example, evaporative light-scattering detector (ELSD) or mass spectrometer (MS) [93]. As it was described in paper by Lima and Abdalla [93] evaporative light-scattering detector is sensitive to mass of vaporized analyte and its operation system is not limited by the absorption characteristics of the individual components and nature of the eluent. Thus, ELSD may be used to solve the problem of separation of fatty acids which have weak absorbance groups [93].

3.2. Mass Spectrometry Detection of Fatty Acids. Application of HPLC-MS in analysis of fatty acids is known only from the last decade. Mass spectrometer is extensively used in analysis of fatty acids especially in case of biological samples. One of the main advantages of HPLC-MS method is possibility to analyze nonvolatile compounds including fatty acids [93]. Numerous ionization and detection modes can be applied for fatty acids analysis such as electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and also time of flight (TOF). A new possibility in HPLC-mass spectrometry is tandem MS (HPLC-MS-MS) or combination of electrospray ionization with tandem mass spectrometer such as HPLC-ESI-MS-MS technique. The major advantage of LC-ESI is the great separation of fatty acids in complex matrices like blood plasma. HPLC-MS-MS is a powerful tool in determination of double bond position or branched points in the chain of unsaturated fatty acids [93]. Next HPLC method developed for detection of long-chain fatty acids is HPLC-MS method combined with APCI, which was applied in detection

of very long-chain fatty acids in form of picolinyl esters coming from sugar cane wax [102]. HPLC-MS coupled with three ionization systems, electron impact (EI), atmospheric pressure chemical ionization, and electrospray ionization, was suitable for the accurate determination of PUFA and also their oxidative metabolites such as eicosanoids in biological samples in the range of pg [103]. According to papers prepared by Řezanka et al., which were focused on fatty acids analysis in lower organisms, liquid chromatography mass spectrometry coupled with ionization mode APCI was the most efficient method applied for identification and quantification of very long-chain polyunsaturated fatty acids from marine organisms (which have ability to produce VLC-PUFA) such as marine dinoflagellates *Amphidinium carterae* and green algae *Chlorella kessleri* [81, 104–106] and also in oil obtained from *Ximenia* fruits (raw material for cosmetic industry) [107]. As it was performed in these papers HPLCMS-APCI system consisted of Hichrom column (HIRPB-250AM) and gradient solvent program with acetonitrile (MeCN), dichloromethane (DCM), and propionitrile (EtCN) which were suitable for separation 13 of picolinyl esters of VLCPUFA from lower organisms. Excellent separation of methyl esters of unsaturated fatty acids coming from freshwater crustacean was achieved by the same mobile phase and the chromatographic column packed with octadecylsilyl phase (Supelcosil LC-18) [108]. An extensive review of a very long polyunsaturated published by Řezanka and Sigler indicates that the modern analytical methods such as HPLC-MS make the detection and identification of VLC-PUFA possible in different classes of lipids including microbial kingdoms and fungi [109]. Reversed-phase HPLC with gradient elution of solvent system containing acetonitrile and chloroform and equipped with light-scattering detector (ELSD) was used to purify and identify the methyl esters of C16–C28 PUFA including octacosaoctaenoic acid in milligram quantities from marine microalgae [82]. A simple method based on reversed-phase ion-pair high performance liquid chromatography (RP-HPLC) was used successfully to separate the various monoepoxides of eicosatrienoic, arachidonic, eicosapentaenoic, and docosahexaenoic acids [110]. These compounds were easily identified by liquid chromatography mass spectrometry (HPLC-MS) with atmospheric pressure chemical ionization (APCI) in nanogram range [110]. This work demonstrated that the method based on APCI-MS coupled with HPLC is highly reliable for the analysis of various monoepoxides of PUFA in their metabolism study. Another HPLC system such as nonaqueous reversed-phase high performance liquid chromatography (NARP-HPLC) with atmospheric pressure chemical ionization (APCI-MS) was suitable for detection of 5 unsaturated polymethylene interrupted fatty acids such as *cis*-5,9-octadecadienoic (taxoleic), *cis*-5,9,12-octadecatrienoic (pinolenic), *cis*-5,11-eicosadienoic (keteleeronic), and *cis*-5,11,14-eicosatrienoic acids (sciadonic) isolated from conifer seed oils (obtained from European Larch, Norway Spruce, and European Silver Fir) [111]. Combination of two chromatographic methods such as TLC and HPLC was used to analyze n-3 fatty acids in fish oil dietary supplements. The EPA and DHA fraction obtained by means of Ag-TLC method were further analyzed by HPLC. The chromatographic column ODS (3.9 mm × 30 cm, 10 μm), mobile phase containing tetrahydrofuran-acetonitrile-water-acetic acid 25 : 35 : 75 : 0.4 (v/v/v/v), and photodiode array detector in the range of 190–240 nm were used in this analysis [112]. HPLC system equipped with analytical column Supelcosil C18 (4.6 mm × 25 cm) and photodiode array detector (DAD) was used for the analysis of hydroperoxy PUFA as the products of peroxidation coming from human plasma. A mixture of acetic acid-acetonitrile-tetrahydrofuran 52 : 30 : 18 (v/v/v) as mobile phase was used. The analysis was monitored at 200–300 nm. Quantity control by applied procedure of obtained derivatives of PUFA may be useful as clinical markers of oxidative stress on biological systems [113]. In another paper gradient reversed-phase liquid chromatography (C18 ODS 25 cm × 0.46 cm, 5 μm) by use of methanol water and universal ELSD detector allowed for separation and fractionation of saturated, unsaturated, and oxygenated free fatty acids as methyl esters extracted from seeds of *Crepis alpina* and *Vernonia anthelmintica*, respectively [114]. This work showed that reversed-phase C18 was suitable for purification and fractionation of PUFA before their quantification by GC-MS. Besides HPLC system equipped with above-mentioned types of detector modes such as ECD, MS, ELSD, DAD, and UV, a fluorescence detector is also very useful [115, 116] which enabled precise determination of long-chain polyunsaturated fatty acids including linolenic, arachidonic, eicosapentaenoic, and docosahexaenoic acids in human serum at low concentrations like fmoles. For example, the method described in 1986 by Yamaguchi et al. performed the conversion of LC-PUFA into fluorescent derivatives by reaction with 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone. Separation process was performed on a reversed-phase column (YMC Pack C8) with an isocratic elution using aqueous 72% (v/v) acetonitrile [115]. Current literature shows that HPLC with different solvent systems is suitable in analysis of novel hydroxyl fatty and phosphoric acid esters of 10-hydroxy-2-decenoic acid (9-HAD) coming from the royal jelly of honeybees (*Apis mellifera*). HPLC analysis was carried out on Diaion HP-20, Sephadex LH-20, and Cosmosil 75C18-OP column systems. Different mobile phases containing acetonitrile were applied. A refractive index detector was applied to monitor the elution profile of examined fatty acids [117].

3.3. Silver Ion HPLC in Quantification and Identification of Geometric Isomers of Fatty Acids. As it was reported by Nikolova-Damyanova and Momchilova [118], silver ion HPLC (Ag-HPLC) is widely applied by many scientists as a preliminary step for the fractionation of complex mixture of fatty acids into their groups prior to quantitative analysis by GC method. Resolution of fatty acids by means of Ag-HPLC is achieved according to the number and geometry of double bonds [118]. Separation is based on the reversible formation of a weak charge-transfer complex between a silver ion and a double bond. The main problem in Ag-HPLC is introduction of Ag^+ into this system. Thus, similar to the case of Ag-TLC the first attempt was performed on column which was laboratory packed with slurry of the stationary phase (e.g., silica gel) impregnated with $AgNO_3$. Second method is

to add silver ion solution ($AgNO_3$) into mobile phase. Third method is to use commercially available silica based cation exchange column (Nucleosil 5 SA), or the column which is produced by Chrompack (ChromSpher 5 lipids). These commercial column systems give much better results (better reproducibility) of fatty acids analysis than those laboratory prepared [118]. Another excellent review of the chromatographic methods used to analyze geometrical and positional isomers of fatty acids by Aini et al. [119] showed that the following factors affect resolution of fatty acids in silver ion HPLC such as the impregnation method of column, mobile phase composition, and also column temperature [119]. The choice of mobile phase composition used in Ag-HPLC is usually toluene based, acetonitrile based, hexane based, or dichloromethane based [118, 120]. Good results have been achieved by the use of isopropanol and tetrahydrofurane as a modifier. The lower column temperature generally results in shorter elution time in Ag-HPLC. Moreover, the improvement in resolution of monoenoic and polyenoic fatty acids was obtained by converting them into phenacyl, benzyl, n-propyl, n-butyl, and ethyl isopropyl esters [52]. Separation by Ag-HPLC coupled with UV detector of *cis*- and *trans*-octadecanoic acids described by Momchilova and Nikolova-Damyanova [83] indicated that the efficiency of the separation increases in the following order: phenethyl < phenacyl < p-methoxyphenacyl esters. Yet, retention and resolution of these fatty acids by Ag-HPLC could be affected by small changes of dichloromethane in mobile phase. Among various chromatographic systems the best resolution of *cis*- and *trans*-positional isomers of octadecenoic acid after converting them into p-methoxyphenacyl esters was achieved on a silver ion column by isocratic elution with a mobile phase containing hexane-dichloromethane-acetonitrile in volume composition of 60 : 40 : 0.2 (v/v/v) [120]. Similar mobile phase was used by Momchilova and Nikolova-Damyanova in 2000 to estimate the chromatographic properties of positional isomers of octadecenoic fatty acids after conversing them to 2-naphthyl, 2-naphthylmethyl, and 9-anthrylmethyl derivatives [83]. According to the results obtained in this paper, dichloromethane-acetonitrile 100 : 0.025 (v/v) as mobile phase provided better resolution of 9-anthrylmethyl derivatives of 6-, 9-, and 11–18 : 1. High resolution by Ag-HPLC was obtained for the positional isomers of PUFA such as eicosapentaenoic acid, docosahexaenoic acid, and also docosapentaenoic acid coming from triacylglycerols containing PUFA. The hexane-isopropanol-acetonitrile solvent system was suitable for the separation of these compounds [84]. Isolation of some PUFA from edible oils by argentate silica gel chromatography (Ag-TLC and Ag-HPLC) was performed by Guil-Guerrero et al. [121]. Using this method the isomers of the following fatty acids, linoleic acid, α-linolenic, γ-linolenic, and stearidonic acid, and eicosapentaenoic and docosahexaenoic acid, have been isolated in form of methyl esters from linseed, sunflower, and borage seed oils and from shortfin mako liver oil. Similarly, like it was described in the previous part, Ag-HPLC was useful for analysis of eicosapentaenoic and docosahexaenoic acid geometrical isomers formed during fish oil deodorization [63]. Obtained results showed that this technique cannot be used to determine the isomers in fish oil which have been formed at the temperature higher than 180°C (e.g., at 220°C), because the interference between obtained isomers, especially di-*trans* DHA and all-*cis* EPA, was observed. Efficient analysis of *trans* isomers of conjugated linoleic acid in synthetic and animal products (from pigs, chicken meat) was achieved also by chromatographic techniques including Ag-HPLC [62]. Recent literature indicates that quantification of separated by Ag-HPLC geometric isomers of fatty acids is mainly performed by GC-MS or by GC coupled with FID (flame-ionization detector). FID, UV, and refractive detector are usually used for direct quantification of isomers of fatty acids as methyl esters. As it was reported by Nikolova-Damyanova and Momchilova [118] aromatic esters of fatty acids can be detected by Ag-HPLC combined with UV-VIS detector in the range of 0–200 μg. The paper prepared in 2013 by Sun and coworkers demonstrates that a liquid chromatography/in-line ozonolysis/mass spectrometry (LC/O_3-MS) is a practical and easy to use new approach to direct determination of double bond position in lipids presented in complex mixture. In order to test this method in complex lipid extracts, a sample of bovine fat with known amount of positional isomers and *cis*/*trans* isomers of unsaturated fatty acids was analyzed. The main advantage of the in-line ozonolysis is its applicability in combination with various mass spectrometers without instrumental modification [122].

Survey of some selected HPLC conditions useful for separation and identification of MUFA and PUFA from various samples is listed in Table 2.

4. Gas Chromatography (GC)

Among different chromatographic methods such as TLC or HPLC, which are described in detail in this paper, gas chromatography (GC) is the most popular for fatty acids analysis. As it was reported by Casal and Oliveira, GC analysis of fatty acids is the first chromatographic method known in fatty acids analysis for 60 years [123]. From this time to 2002 year numerous original and review papers were performed with emphasis on a gas chromatographic analysis of the common and uncommon fatty acids [85, 123–125].

The aim of our work was to describe present-day GC analysis of fatty acids belonging to mono- and polyunsaturated group (MUFA and PUFA), whose biological significance has increased in recent years. This paper was prepared on the basis of the papers coming from the last decade (2002–2013).

4.1. Derivatization Methods for GC Analysis of Fatty Acids. The main step in GC analysis of fatty acids is the process of conversion of these compounds into suitable volatile derivatives [123]. The most frequently applied derivatives are alkyl derivatives (methyl-, ethyl-, and isopropyl-) obtained by means of esterification of fatty acids. This procedure facilitates volatility of fatty acids and also improves their separation and sensitivity to GC detectors. For this aim, the following esterification methods are applied [123]: methylation catalyzed by H^+, esterification catalyzed by OH^-, esterification with benzyl alcohol (p-bromobenzyl, p-methylbenzyl, and p-nitrobenzyl iodide), methylation with diazomethane in

Table 2: Survey of some selected HPLC conditions useful for separation and identification of MUFA and PUFA from various samples.

HPLC system	Mobile phase	Column	Detection mode	Matrix	Remarks	Reference
Ag-HPLC	Isocratic solvent system: hexane-acetonitrile 99.7 : 0.3 (v/v)	Chromspher 5 Lipid column (250 × 4.6 mm, Varian, Netherlands)	UV-VIS detector	Deodorized fish oil containing EPA, DHA, GLA, LA, ALA, SD, and other PUFA	Detection level[a]	[63]
HPLC-ECD	Ethanol-acetonitrile 20 : 80 (v/v)	ODS 100RP-18 column, LiChrospher 250 mm × 4 mm, 5 μm phase particle	Electrochemical detector, saturated calomel electrode, detection potential −415 mV	Human plasma, containing various fatty acids including arachidonic acid and linoleic and oleic acids	Detection level: 50–1600 pmoles	[80]
HPLC-MS-APCI	The gradient solvent program was used: MeCN/EtCN/DCM 60 : 30 : 10 (v/v/v) MeCN/EtCN/DCM 30 : 40 : 30 (v/v/v)	Hichrom column HIRPB-250 AM, 250 mm × 2.1 mm, 5 μm phase particle	Picolinyl esters were separated by use of quadruple mass spectrometer fitted with an atmospheric pressure chemical ionization source	Dinoflagellate *Amphidinium carterae* containing VLC-PUFA up nonacosahexaenoic acid	Detection level[a]	[81]
RP-HPLC-ELSD	The isocratic elution of MeCN/H_2O was used: 97.5 : 2.5 (v/v) flow rate of mobile phase 1.5 ml/min	RP column (Alltima, C18, Alltech, 250 mm × 4.6 mm), 5 μm phase particle	Methyl esters PUFA were detected by evaporative light-scattering detector (ELSD)	Marine algae *Schizochytrium* sp. containing DHA, EPA, and their derivatives	Detection level: mg	[82]
Ag-HPLC	Dichloromethane-acetonitrile 100 : 0.025 (v/v)	Nucleosil 100-5SA (250 mm × 4.6 mm); Hichrom, Reading, UK	UV-VIS detector, the working wavelength was 250 nm for the anthrylmethyl and naphthyl derivatives and also 275 nm for the naphthylmethyl derivatives of examined octadecenoic acid	Reference standards of positional isomers of octadecenoic acid and their derivatives (esters)	Detection level: μg	[83]
Ag-HPLC	Gradient solvent system consists of hexane-isopropanol-acetonitrile 350 : 100 : 2.75 (v/v/v) 350 : 100 : 10 (v/v/v)	Chrompack 5 Lipid column (250 mm × 4.6 mm, Netherlands)	UV-VIS detector, the working wavelength was 206 nm APCI-MS	Oil produced by a marine microbe containing PUFA (DHA, EPA)	Detection level[a]	[84]

[a] Not described by authors; MeCN-acetonitrile, EtCN-propionitrile, and DCM-dichloromethane.

ethereal solution, or with boron trichloride (BCl_3-MeOH), methylation by means of tetramethylammonium (TMAH) hydroxide or trimethylsulfonium hydroxide (TMSH), derivatization with *tert*-butyldimethylsilyl (tBDMSi) or dimethyloxazolidine (DMOX), and also cyanomethyl derivatization.

Among the above-presented derivatization procedures the most important for GC-MS analysis of mono- and polyunsaturated fatty acids are DMOX derivatives and picolinyl derivatives which are widely applied for the determination of double sites in unsaturated fatty acids. A rapid and inexpensive method for separation of very long-chain fatty acids is cyanomethyl derivatization.

4.2. Instrumentation of GC. In gas chromatography of fatty acids, a variety of columns with different properties such as polar and nonpolar are available. Of all the most frequently applied in fatty acids analyses are the polar columns. The fused silica columns allow improvements in the separation of PUFA, for example, from fish oil samples. Another type of stationary phases used for fatty acids analysis is polar polyesters such as Carbowax column, for example, PEG (polyethylene glycol) which is commercially available as Carbowax-20 M, CP-Wax 52CB, DB-Wax, and so forth. Next example of polar stationary phase used in GC is cyanopropyl polysiloxane such as commercially available HP-88, CP-Sil88, BPX70, SP-2340, and SP-2560. As it was described in Casal and Oliveira report [123], the high polarity of the cyanosilicone phases allows the separation of geometrical isomers (*cis* and *trans*) of mono- and polyunsaturated fatty acids [123]. The literature review indicates that packed GC columns (glass tubing) with stationary phase containing usually from 6 to 5 mm diameters are still in use in fatty acid analysis. Among them the most popular is Chromosorb series. Others, which belong to polar stationary phases, are organosilicon polyesters (EGSS-X), butanediol succinate (BDS), and diethylene glycol succinate (DECS) [121, 123].

Of different GC detectors which show high sensitivity (from nanograms to femtograms) such as NPD (nitrogen phosphorus-sensitive detector), FDP (flame photometric detector), ELCD (electrolytic conductivity detector), and PID (photoionization detector), FID (flame ionization detector) and also mass detector (MS) are commonly used in fatty acids analysis [124]. FID allows detecting the organic substances including unsaturated fatty acids mono- and poly- in picograms. The carrier gases used in this method are nitrogen, helium, and hydrogen. GC-MS detection is a powerful tool in GC analysis of fatty acids (saturated and unsaturated) coming from different sources such as sea water, wood, and fish oil. The fact that MS is suitable for the localization of double bond in polyunsaturated fatty acids is important. An efficient detection method in study of very long-chain fatty acid esters in biological sample (blood, urine) and wax is tandem spectrometry MS/MS [18]. Recently, a novel trend in GC detection is coupling two different detectors in parallel to the outlet of column, for example, FID-ELCD. Moreover, application of GC analysis with FT-IR or NMR technique can facilitate the determination of functional groups and structural studies of fatty acids in the case of their geometrical isomers [18].

4.3. GC-Mass Spectrometry (GC-MS) of Fatty Acids. Besides GC-FID, mass spectrometry coupled to GC (GC-MS) is the most powerful tool for identification of mono- and polyunsaturated fatty acids. This technique requires derivatization of them into picolinyl esters, pyrrolidines, or 4,4-dimethyloxazolidines derivatives, respectively [123]. Casal and Oliveira confirmed that the picolinyl esters are very suitable in MS analysis of MUFA and PUFA because they offer a specific mass fragmentation pattern [123]. In 2005 Destaillats et al. have shown that methyl ester derivatives used for routine quantification by GC-FID can be also used for structural investigation by GC-MS of long-chain polyunsaturated fatty acids (e.g., metabolites of rumelenic acid) [85]. An improved method of GC-MS for the identification of very long-chain highly unsaturated fatty acids with more than 22 carbon atoms (C_{28}) such as octacosaheptaenoic acid (28 : 7, n-6) and octacosaoctaenoic acid (28 : 8, n-3) in seven marine dinoflagellate species was developed by Monsour et al. [86]. In another work, the total lipids of three crustacean species coming from Lake Baikal were analyzed for long-chain polyunsaturated fatty acids such as 24 : 3 (n-6), 24 : 3 (n-3), 24 : 5 (n-3), 26 : 3 (n-6), 26 : 5 (n-6), and also 28 : 7 (n-6) by use of Ag-TLC and GC-MS [87]. In further study, automated GC-MS was applied to identify polyunsaturated fatty acids in algal and microbial cultures (some freshwater algae and in filamentous cyanobacterium). A novel GC-MS method developed by Akoto et al. in 2005 enabled detection the polyunsaturated fatty acids after their previous derivatization into corresponding methyl esters. By means of the described method unique PUFA in some green algae and cyanobacterium, which play a role of biomarkers in ecological and food studies, can be determined [88]. Another work demonstrated that GC-MS can be successfully used for quantitative analysis of total n-3 and n-6 fatty acids in human plasma. Six PUFA were esterified and next quantified by the use of GC-MS and also by means of reference spectrophotometric method. GC analysis was performed on DB-23 fused silica column (30 m × 0.32 mm, 0.25 mm). Helium was applied as carrier gas. This study showed that applying of GC-MS in quantification of PUFA (n-3 and n-6) gives the results with the high accuracy. In addition, the statistical evaluation of both analytical methods indicated that there is no significant difference between GC-MS and spectrophotometric method [126]. In the next paper, the content of seventy-two unsaturated fatty acids (polyenoic) present in mantle, muscle, and viscera of the marine bivalve *Megangulus zyonoensis* was determined [127]. The GC analysis coupled to FID was performed on capillary column (Supelco, 30 m × 0.32 mm, 0.25 mm). Helium was used as carrier gas at a flow rate of 1.5 mL/min. DMOX derivatives of examined fatty acids were analyzed by means of GC-MS equipped also with Omegawax-320 capillary column (Supelco, 30 m × 0.32 mm, 0.25 mm). The spectra were recorded at ionization energy of 70 eV. Another example indicates the usefulness of GC-MS in analysis of a new octadecatrienoic acid as a component of *Chrysanthemum zawadskii Herb*. (seed oil). To confirm the structure of the new unusual fatty acids different chemical derivatizations (preparation of methyl esters, pyrrolidine, picolinyl esters, and dimethyloxazolidine

derivatives) were applied [128]. Capillary GC analysis was carried out using GC chromatograph equipped with FID and a 50 m × 0.25 mm column CP-Sil 88 by means of hydrogen as a carrier gas at a flow rate of 2 mL/min. GC-MS analysis of fatty acid derivatives were performed on silica fused column Supelcowax 10 (30 m × 0.25 mm, 0.25 μm). Helium was used as a carrier gas [128]. The reviewed papers showed that a rapid GC-MS method is the most frequently used technique for the quantification and identification of geometric isomers of long-chain PUFA including CLA [129]. A series of CLA with double bond positions with all possible geometries (*cis/trans, trans/cis, cis/cis,* and *trans/trans*) were converted into methyl esters and next chromatographically analyzed in the low femtomole range for diagnostic purpose (in clinical study) [130]. Performed GC method should be more accurate to direct quantification of total *trans*-octadecenoic acid in food samples (e.g., milk). Results presented by Destaillats et al. [131] confirmed that the isomeric profile of *trans*-octadecenoic acid in milk fat determined by GLC in combination with Ag-TLC may be found as the reference method.

The recently published paper by Liu and coworkers indicates that among various analytical techniques GC-MS method using newer generation of detection system such as single quadrupole mass detector is high sensitive new tool for studies of n-3/n-6 PUFA in small samples of human and mouse retina. These studies suggest that the method developed by Liu et al. could be satisfactorily applied in the treatment of the pathological process of macular degenerations and dystrophies [132].

4.4. GC-FID Analysis (Gas Chromatography Coupled with Flame Ionization Detector) of Fatty Acids. The current research shows that flame ionization mode of detection is the most common analytical method used in routine quantitative analysis of fatty acids in various matrices, because it is robust and of low cost in comparison with mass spectrometry [123]. The studies conducted by Schreiner confirmed the accuracy and robustness of GC-FID technique for quantification of LC-PUFA [133]. In order to check the robustness of gas chromatographic method in combination with FID for quantification of PUFA, various chromatographic systems were used by Schreiner. Thus, this work is a guideline which explains how to achieve the accuracy 17 and robustness of GC-FID method. During the last decade there is an increase in the application of GCFID in monitoring of omega-3 and omega-6 fatty acids in various matrices samples including human and nutritional. According to Böcking and coworkers PUFA including the two presented above fatty acids and linoleic acid could be satisfactorily quantified using GC-FID in serum for clinical diagnosis (e.g., in case of cardiovascular diseases) and in quality control of food, for example, cow's milk [89]. Another example of application of GC-FID in analysis of biological samples shows the paper performed by Bláha et al. [134]. The described GC-FID study of selected MUFA and PUFA (e.g., CLA, EPA, and DHA) and their metabolites were analyzed in human blood under the following conditions: fused silica capillary column SP2330 (30 m × 0.25 mm), helium as a carrier gas, and temperature of column in the range from 120°C to 230°C. The developed method plays a significant role in coronary atherosclerosis study. Quantitative aspect of GC-FID bioanalysis of PUFA in plasma lipoproteins was also widely described by Tvrzická and coworkers [135]. Novel possibilities in GC-FID analysis are to use this technique for determining of medium and long-chain polyunsaturated fatty acids in clinical (parenteral) formulations [136]. The data presented in this paper indicate that GC-FID can be easily used for accurate determination of PUFA composition in lipid emulsion. Most of the papers regarding the use of gas chromatography for analysis of polyunsaturated fatty acids such as linoleic acid and their geometrical isomers present in dairy fat (e.g., milk fat) recommend the column type Carbowax such as Supelcowax 10 [137]. The main advantage of this column is a better resolution of the linolenic and eicosadienoic acid isomers [137]. Kramer and Cruz-Hernandez and their coworkers indicated that besides polyethylene glycol capillary column, 100-m CP-Sil 88 column (100% cyanopropyl polysiloxane) could be very suitable in PUFA analysis including CLA isomers in milk fat [76, 137]. Both helium and hydrogen were used as the carrier gases, respectively. Gas chromatograph was equipped with FID. The profile of polyunsaturated fatty acids especially *trans* isomers in food samples is usually analyzed by gas chromatography. The official method recommends the use of fatty acids in form of methyl esters [138]. In 2000 Timmins and coworkers developed a method for quantification of omega-3 from marine oils as ethyl esters. This analysis was conducted with gas chromatograph coupled with FID on an Omegawax 320 fused silica capillary column (30 m × 0.32 mm, 0.25 μm). The carrier gas was helium at a pressure 63 kPa [139]. Profile of polyunsaturated *trans* fatty acids in food products (e.g., peanut oil) including preparation of fatty acid methyl esters was successfully determined with a highly polar column CP-Sil 88. Moreover, the methylation procedure allows accurate isolation of geometrical isomeric of linolenic acid [140]. Tyburczy et al. studied the *trans* polyunsaturated fatty acids in selected fast food such as hamburger, pizza, chicken, cheese, and others [90]. This fact shows usage of GC-FID method in monitoring of the *trans* fatty acid level in some restaurant foods. The current research indicates the usefulness of GC-FID in quantification of some mono- and polyunsaturated fatty acids (e.g., oleic, linoleic, and linolenic) in oils of wheat germ and seeds produced as by-products [91]. The content of long-chain polyunsaturated fatty acids like eicosapentaenoic acid and docosahexaenoic acid in different assortments of smoked Atlantic mackerel and Baltic sprats was determined by the use of gas chromatograph equipped with FID [92]. The results demonstrate that the applied GC-FID system is a powerful analytical tool in control of the PUFA content in sprats smoked fish which are the source of n-3 and n-6 fatty acids. A recent paper has shown GC-FID determination of fatty acid methyl esters in biodiesel bland using an ionic liquid stationary phase [141]. In this work the two 1,9-di(3-vinyl-imidazolium)nonane bis(trifluoromethyl)sulfonylimidate stationary phase capillary columns SLB-IL-100 (30 m × 0.25 mm, 0.25 μm) and on SLB-IL-100 (12 m × 0.10 mm, 0.08 μm) have been evaluated to determine the profile of fatty acids including eicosanoic, linoleic, oleic, and linolenic in biodiesel blend.

4.5. Gas Chromatography in Analysis of Geometrical and Positional Isomers of Fatty Acids. As it was described in previous section regarding TLC and HPLC methods, the analysis of geometrical isomers and position of double bonds may give a great impact on nutritional study, especially in food quality control. To obtain good results in analysis of geometrical isomers of mono- and polyunsaturated fatty acids, they should be converted into suitable derivatives. To determine the number of double bonds the GC equipped with MS detector is enough [119]. Among various derivatives, those which ensure good results in identification of double bond position by GC-MS technique are 4,4-dimethyloxazolidine (DMOX) derivatives and picolinyl esters [119]. In the case of monoenoic acids conversion into dimethyl disulphide is satisfactory. In addition, identification of fatty acids double bond position is possible by GC coupled with MS/MS detector which requires the derivatization of the fatty acids into proper compounds, for example, PTSA derivatives (p-toluenesulfonic acid derivatives) [142]. To the most popular stationary phases which enable satisfactory resolution and positional characteristic of double bonds in unsaturated fatty acids belongs cyanopropyl capillary column such as CP-Sil 88 in case of MS/MS detection or Supelco 2560 capillary column which is suitable for GC-FID analysis [141].

4.6. GC × GC (Two-Dimensional Gas Chromatography). Two-dimensional gas chromatography is a new chromatographic technique which recently plays a very important role in resolution of fatty acids in complex samples such as lipids coming from marine biota (e.g., algae). According to Casal and Oliveira, the separation process by two-dimensional gas chromatography (2D-GC) is carried out on the two columns with different polarity [123]. For example, in 2011 Gu and coworkers performed a comprehensive two-dimensional analysis of fatty acids including PUFA in marine biota [143]. The results described in this paper show that, in GC × GC method using an apolar polydimethylsiloxane × polar ionic liquid column combination, an excellent resolution of unsaturated fatty acids complementary to GC-MS identification was achieved. Another work performed by Manzano and coauthors [144] confirmed that GC × GC system using an apparatus equipped with capillary flow technology and a FID has been satisfactorily used to separate a large number of fatty acids including the eight isomers of the linolenic acid and also the linoleic isomers. All examined fatty acids were obtained from broccoli leaves by use of supercritical fluid extraction (SFE). The results of this experiment showed that SFE technique and GC × GC analysis with FID or MS detector are suitable to study fatty acids [144]. The current literature in emphasis on mono- and polyunsaturated fatty acids analysis in different matrix indicates that besides the widely used chromatographic techniques such as TLC, HPLC, and GC equipped with various detectors, the relatively new method is gas chromatography coupled to isotope ratio mass spectrometer (known as IRMS) [145]. As it was reported by Meier-Augenstein [145], this technique may be used in fatty acids analysis in complex biological samples and it is alternative to the typical detection systems applied in chromatographic analysis. Another new solution in gas chromatographic analysis of unsaturated fatty acids is to use GC in combination with infrared spectroscopy based on transmission (GC-IR) or on attenuated reflection (GC-ATR) [146]. Both techniques are refined in quantification of *trans* fatty acids in food products (margarines, vegetable oils).

Survey of some selected GC conditions useful for separation and identification of some MUFA and PUFA from different matrices is listed in Table 3.

5. Supercritical Fluid Chromatography (SFC)

A brief review of the chromatographic studies of LC-MUFA and LC-PUFA indicated that, among traditional GC technique and HPLC method, an alternative or complementary method to both of the mentioned techniques is supercritical fluid chromatography (SFC). Supercritical fluid chromatography is known as hybrid of gas and liquid chromatography. In contrast to GC and HPLC, SFC allows the use of higher flow rates, low temperature, no derivatization, and also universal detectors such as FID, ELSD, or MS [147]. This method leads to greater efficiency in short time analysis times and reduced consumption of organic solvents. Moreover as was accurately described by Señoráns and Ibañez [148] it permits the separation and determination of thermally labile (e.g., polyenic fatty acids), low volatile, and polar fatty acids (e.g., hydroxy fatty acids) which cannot be analyzed by HPLC or GC without derivatization. For this reason, in the last few years the use of SFC in fatty acid analysis has attracted increasing interest in the food sector [147, 148].

Supercritical fluid chromatography was usually performed with pure carbon dioxide (CO_2) as the mobile phase. Nowadays SFC is often carried out in subcritical conditions, and in many cases CO_2 is modified with an organic solvent in order to increase the solubility of polar compounds [147–149].

SFC separation of fatty acids has been performed by means of different chromatographic columns, such as open tubular, packed, and packed capillary. The open tubular columns are preferred for separations when high efficiencies are required and also for complex samples [147–149].

Different detection modes are applied in SFC analysis such as UV-VIS detectors, ELSD (evaporative light scattering detector), and MS detector (mass spectrometer), but the most widely used in SFC is MS detector. It was stated that SFC analysis combined with MS detector provided very good levels of sensitivity. Similar to liquid chromatography (HPLC-MS), atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) are the most popular ionization methods recommended for SFC-MS analysis. Moreover, there is no limitation of mass analyzer that could be coupled to SFC system [147–149].

All important parameters such as mobile phase composition, columns, and the detection mode systems needed to SFC analysis of fatty acids were accurately described in review papers by Señoráns and Ibañez in 2002 and also by Bernal and coworkers in 2013 [148, 149]. Excellent review study by Bernal et al. of the analysis of fatty acids in foods demonstrates that new trend in SFC analysis is SFC × SFC system. The use of two-dimensional SFC (2D-SFC) or SFC coupled to RP-HPLC improves the separation of very complex mixtures.

Table 3: Survey of some selected GC conditions useful for separation and identification of MUFA and PUFA from various samples.

Analyte	GC column	Derivatization	Identification/quantification	Reference
Liver lipids from rat containing rumelenic acid (*cis*-9, *trans*-11, and *cis*-15 18 : 3) isomer of linolenic acid	BPX-70 capillary column SGE, Melbourne (50 m × 0.32 mm, 0.25 μm), helium was used as carrier gas under flow 2.4 mL/min	4,4-dimethyloxazolidine (DMOX) and methyl ester derivatives	Selective quadrupole mass detector under ionization voltage of 70 eV at 230°C	[85]
The algae extract containing 28 : 7 (n-6) and also 28 : 8 (n-3)	Polar column: BPX-70, (50 m × 0.32 mm, 0.25 μm), nonpolar: HP-1 capillary column (50 m × 0.32 mm, 0.17 μm) helium was used as carrier gas	DMOX derivatization	Mass spectrometer (Fisons, MD-800) under ionization voltage of 35 eV at 200°C	[86]
Extracts from three crustacean species coming from Lake Baikal containing n-3 and n-6 PUFA	Supelcowax 10 column (60 m × 0.25 mm, 0.25 μm), the temperature program was from 100°C to 280°C. The carrier gas was helium at flow rate of 70 cm/s	Methyl esters and oxazolines of PUFA	Mass spectrometer with chemical ionization (Finnigan MAT 1020B)	[87]
Extracts from green algae and cyanobacterium	A fused HP-5MS (Agilent) capillary column (30 m × 0.25 mm, 0.1 μm), helium was used as mobile phase	Methyl esters of PUFA	Ion trap mass spectrometer (ITD-MS) was used	[88]
Clinical formulations containing PUFA	Capillary column Zebron ZB-WAX plus (30 m × 0.25 mm, 0.25 μm)	Methyl esters of PUFA	GC-FID system	[89]
Food samples containing saturated and unsaturated fatty acids (MUFA and PUFA) *cis* and *trans*	SP-2560 CPS column (30 m × 0.25 mm, 0.20 μm), hydrogen was used as a carrier gas	Methyl esters of MUFA and PUFA	GC-FID system	[90]
Oil extracts from grape seed and wheat germ rich in linolenic and linoleic acids	DB-23 (0.32 mm × 30 m), temperature programming 150–230°C, carrier gas was helium	Methyl esters of linolenic and linoleic acid	GC-FID system	[91]
Smoked mackerel meat containing EP and DHA	Column Rtx 2330 (105 m × 0.25 mm, 0.2 μm) temperature programming 30–210°C	Methyl esters of EP and DHA	GC-FID system	[92]

A preparative supercritical fluid chromatography was applied for PUFA separation to obtain enriched fractions of EPA from fish oil mixture. Silica gel as the stationary phase and carbon dioxide as the supercritical fluid were used. Under these conditions the best purity in EP-EE (eicosapentaenoic ethyl ester) reaches 95% with a 24.6% yield [149]. Recent metabolomic investigations show that the SFC-MS enables the simultaneous rapid analysis of lipids with varied structure. Applying cyanopropyl bonded silica gel-packed column the resolution of lipid mixture from the leaf of *Catharanthus roseus* resulted in satisfactory separation of lipid mixture with time less than 15 minutes. The data obtained in this study confirm that SFC system is a powerful tool for studies on lipid metabolomic because it is useful as a fingerprinting method not only for the screening of various lipids but also for the detailed profiling of their individual components [150, 151].

6. Conclusions

This literature review which is focused on the application of the chromatographic methods such as TLC, HPLC, GC, and also SFC in analysis of mono- and polyunsaturated fatty acids (MUFA, PUFA) in different matrices shows that all of the presented chromatographic methods are suitable in preseparation and quantification of these compounds. The choice of the chromatographic systems depends on the type of the sample to be analyzed and on the aim of chromatographic analysis (problem which should be solved by means of this technique). For instance, in order to obtain complete fractionation of "*trans*/*cis*" fatty acids from different samples, the traditional Ag-TLC or argentation HPLC (Ag-HPLC) is used. The column chromatography such as GC and HPLC may be used in further steps of chromatographic analysis of unsaturated fatty acids (MUFA and PUFA), especially to determine the quantity of separated fatty acids. In the case of geometrical isomers (*cis* and *trans*) and determining of the number and position of double bonds present in studied unsaturated fatty acids, Ag-HPLC and various GC systems are popular. Current literature indicates that the innovations which are implemented into chromatographic systems include modified stationary phases, new derivatizing agents for fatty acids, and also developing of novel detection systems allowing sensitive and reproducible fatty acid analysis from complex samples. Moreover, the new chromatographic systems combined with other instrumental methods such as MS, MS/MS, IR, or two-dimensional GC enable analysis of a large number of MUFA and PUFA in lower quantities, which is required, for example, in food analysis. The results presented in our paper confirm that the chromatographic methods are still a powerful tool in analysis of mono- and polyunsaturated fatty acids.

Conflict of Interests

The authors declare no conflict of interests regarding the publication of this paper.

Acknowledgments

This research was financed by the Medical University of Silesia as part of statutory research in 2014 Project no. KNW-1-006/N/4/0.

References

[1] D. Voet and J. G. Voet, *Biochemistry*, John Wiley & Sons, New York, NY, USA, 4th edition, 2010.

[2] R. Murray, K. M. Botham, P. J. Kennelly et al., *Harper's. Illustrated Biochemistry*, McGraw-Hill Education, Toronto, Canada, 29th edition, 2012.

[3] P. Risé, F. Marangoni, and C. Galli, "Regulation of PUFA metabolism: pharmacological and toxicological aspects," *Prostaglandins Leukotrienes and Essential Fatty Acids*, vol. 67, no. 2-3, pp. 85–89, 2002.

[4] A. Sellmayer and P. C. Weber, "Polyunsaturated fatty acids and cardiovascular risk: Interference at the level of gene expression," *The Journal of Nutrition Health and Aging*, vol. 6, no. 4, pp. 230–236, 2002.

[5] L. Arab, "Biomarkers of fat and fatty acid intake," *Journal of Nutrition*, vol. 133, supplement 3, pp. 925S–932S, 2003.

[6] P. Benatti, G. Peluso, R. Nicolai, and M. Calvani, "Polyunsaturated fatty acids: biochemical, nutritional and epigenetic properties," *Journal of the American College of Nutrition*, vol. 23, no. 4, pp. 281–302, 2004.

[7] W. Karmally, "Balancing unsaturated fatty acids: what's the evidence for cholesterol lowering?" *Journal of the American Dietetic Association*, vol. 105, no. 7, pp. 1068–1070, 2005.

[8] O. Ozyilkan, D. Colak, Z. Akcali, and B. Basturk, "Olive: fruit of peace against cancer," *Asian Pacific Journal of Cancer Prevention*, vol. 6, no. 1, pp. 77–82, 2005.

[9] A. Lapointe, C. Couillard, and S. Lemieux, "Effects of dietary factors on oxidation of low-density lipoprotein particles," *The Journal of Nutritional Biochemistry*, vol. 17, no. 10, pp. 645–658, 2006.

[10] J. S. Perona, R. Cabello-Moruno, and V. Ruiz-Gutierrez, "The role of virgin olive oil components in the modulation of endothelial function," *The Journal of Nutritional Biochemistry*, vol. 17, no. 7, pp. 429–445, 2006.

[11] P. D. Biondo, D. N. Brindley, M. B. Sawyer, and C. J. Field, "The potential for treatment with dietary long-chain polyunsaturated n-3 fatty acids during chemotherapy," *The Journal of Nutritional Biochemistry*, vol. 19, no. 12, pp. 787–796, 2008.

[12] B. Koletzko, E. Lien, C. Agostoni, and et al, "The roles of long-chain polyunsaturated fatty acids in pregnancy, lactation and infancy: review of current knowledge and consensus recommendations," *Journal of Perinatal Medicine*, vol. 36, no. 1, pp. 5–14, 2008.

[13] C. Ruggiero, F. Lattanzio, F. Lauretani, B. Gasperini, C. Andres-Lacueva, and A. Cherubini, "Ω-3 polyunsaturated fatty acids and immune-mediated diseases: Inflammatory bowel disease and rheumatoid arthritis," *Current Pharmaceutical Design*, vol. 15, no. 36, pp. 4135–4148, 2009.

[14] J. T. Brenna, N. Salem Jr., A. J. Sinclair, and S. C. Cunnane, "α-Linolenic acid supplementation and conversion to n-3 long-chain polyunsaturated fatty acids in humans," *Prostaglandins Leukotrienes and Essential Fatty Acids*, vol. 80, no. 2-3, pp. 85–91, 2009.

[15] P. A. Corsetto, G. Montorfano, S. Zava, I. E. Jovenitti, A. Cremona, and A. M. Rizzo, "Effects of n-3 PUFAs on breast cancer cells through their incorporation in plasma membrane," *Lipids in Health and Disease*, vol. 10, article 73, 2011.

[16] I. M. Berquin, I. J. Edwards, S. J. Kridel, and Y. Q. Chen, "Polyunsaturated fatty acid metabolism in prostate cancer," *Cancer and Metastasis Reviews*, vol. 30, no. 3-4, pp. 295–309, 2011.

[17] M. Kaczmarski, B. Cudowska, M. Sawicka-Żukowska, and A. Bobrus-Chociej, "Supplementation with long chain polyunsaturated fatty acids in treatment of atopic dermatitis in children," *Advances in Dermatology and Allergology*, vol. 30, no. 2, pp. 103–107, 2013.

[18] I. Brondz, "Development of fatty acid analysis by high-performance liquid chromatography, gas chromatography, and related techniques," *Analytica Chimica Acta*, vol. 465, no. 1-2, pp. 1–37, 2002.

[19] J.-M. Kornprobst and G. Barnathan, "Demospongic acids revisited," *Marine Drugs*, vol. 8, no. 10, pp. 2569–2577, 2010.

[20] C. Bigogno, I. Khozin-Goldberg, S. Boussiba, A. Vonshak, and Z. Cohen, "Lipid and fatty acid composition of the green oleaginous alga *Parietochloris incisa*, the richest plant source of arachidonic acid," *Phytochemistry*, vol. 60, no. 5, pp. 497–503, 2002.

[21] M. A. Hossain, "Fish as source of n-3 polyunsaturated fatty acids (PUFAs), which one is better-farmed or wild?" *Advance Journal of Food Science and Technology*, vol. 3, no. 6, pp. 455–466, 2011.

[22] O. P. Ward and A. Singh, "Omega-3/6 fatty acids: alternative sources of production," *Process Biochemistry*, vol. 40, no. 12, pp. 3627–3652, 2005.

[23] L. A. Copeman and C. C. Parrish, "Lipids classes, fatty acids, and sterols in seafood from Gilbert Bay, southern Labrador," *Journal of Agricultural and Food Chemistry*, vol. 52, no. 15, pp. 4872–4881, 2004.

[24] A. Gupta, C. J. Barrow, and M. Puri, "Omega-3 biotechnology: thraustochytrids as a novel source of omega-3 oils," *Biotechnology Advances*, vol. 30, no. 6, pp. 1733–1745, 2012.

[25] V. Manikan, M. Sahaid Kalil, M. H. Mohd Isa, and A. Abdul Hamid, "Improved prediction for medium optimization using factorial screening for docosahexaenoic acid production by Schizochytrium SP. SW1," *American Journal of Applied Sciences*, vol. 11, no. 3, pp. 462–474, 2014.

[26] D. S. Nichols, "Prokaryotes and the input of polyunsaturated fatty acids to the marine food web," *FEMS Microbiology Letters*, vol. 219, no. 1, pp. 1–7, 2003.

[27] A. Arjuna, "Production of polyunsaturated fatty acids by fungi: a review," *International Journal of Pharma and Bio Sciences*, vol. 5, pp. 931–954, 2014.

[28] S. Das, P. S. Lyla, and S. Ajmal Khan, "Fatty acid profiles of marine benthic microorganisms isolated from the continental slope of Bay of Bengal: a possible implications in the benthic food web," *Ocean Science Journal*, vol. 42, no. 4, pp. 247–254, 2007.

[29] J. E. Karr, J. E. Alexander, and R. G. Winningham, "Omega-3 polyunsaturated fatty acids and cognition throughout the lifespan: a review," *Nutritional Neuroscience*, vol. 14, no. 5, pp. 216–225, 2011.

[30] Y. Abe, M. Honsho, H. Nakanishi, R. Taguchi, and Y. Fujiki, "Very-long-chain polyunsaturated fatty acids accumulate in phosphatidylcholine of fibroblasts from patients with Zellweger syndrome and acyl-CoA oxidase1 deficiency," *Biochimica et Biophysica Acta: Molecular and Cell Biology of Lipids*, vol. 1841, no. 4, pp. 610–619, 2014.

[31] K. Hama, T. Nagai, C. Nishizawa et al., "Molecular species of phospholipids with very long chain fatty acids in skin fibroblasts of Zellweger syndrome," *Lipids*, vol. 48, no. 12, pp. 1253–1267, 2013.

[32] M. P. Agbaga, N. A. Mandal, and R. E. Anderson, "Retinal very long-chain PUFAs: new insights from studies on ELOVL4 protein," *Journal of Lipid Research*, vol. 51, no. 7, pp. 1624–1642, 2010.

[33] M. I. Aveldano, "A novel group of very long chain polyenoic fatty acids in dipolyunsaturated phosphatidylcholines from vertebrate retina," *Journal of Biological Chemistry*, vol. 262, no. 3, pp. 1172–1179, 1987.

[34] L. D. Bennett, R. S. Brush, M. Chan et al., "Effect of reduced retinal VLC-PUFA on rod and cone photoreceptors," *Investigative Ophthalmology & Visual Science*, vol. 55, no. 5, pp. 3150–3157, 2014.

[35] I. A. Butovich, "Lipidomics of human meibomian gland secretions: chemistry, biophysics, and physiological role of meibomian lipids," *Progress in Lipid Research*, vol. 50, no. 3, pp. 278–301, 2011.

[36] B. Nikolova-Damyanova, "Fatty acids: silver ion TLC," in *Encyclopedia of Chromatography*, J. Cazes, Ed., pp. 846–848, Taylor and Francis, New York, NY, USA, 3rd edition, 2010.

[37] J. C. Touchstone, "Thin-layer chromatographic procedures for lipid separation," *Journal of Chromatography B: Biomedical Applications*, vol. 671, no. 1-2, pp. 169–195, 1995.

[38] B. Nikolova-Damyanova, R. Velikova, and L. Kuleva, "Quantitative TLC for determination of the triacylglycerol composition of sesame seeds," *Journal of Liquid Chromatography & Related Technologies*, vol. 25, no. 10-11, pp. 1623–1632, 2002.

[39] B. Nikolova-Damyanova, "Quantitative thin-layer chromatography of triacylglycerols. Principles and application," *Journal of Liquid Chromatography and Related Technologies*, vol. 22, no. 10, pp. 1513–1537, 1999.

[40] T. Nakamura, M. Fukuda, and R. Tanaka, "Estimation of polyunsaturated fatty acid content in lipids of aquatic organisms using thin-layer chromatography on a plain silica gel plate," *Lipids*, vol. 31, no. 4, pp. 427–432, 1996.

[41] B. Fuchs, R. Süß, K. Teuber, M. Eibisch, and J. Schiller, "Lipid analysis by thin-layer chromatography—a review of the current state," *Journal of Chromatography A*, vol. 1218, no. 19, pp. 2754–2774, 2011.

[42] J. Sherma, "Thin-layer chromatography in food and agricultural analysis," *Journal of Chromatography A*, vol. 880, no. 1-2, pp. 129–147, 2000.

[43] B. Fuchs, K. Teuber, A. Nimptsch et al., "Thin-layer chromatography with direct mass spectrometric analyte detection—a review of the progress achieved in the last five years," in *High-Performance Thin-Layer Chromatography (HPTLC)*, M. M. Srivastava, Ed., pp. 335–363, Springer, New York, NY, USA, 2011.

[44] B. Fuchs, A. Nimptsch, R. Süss et al., "The analysis of brain lipids by a directly coupled MALDI/TLC approach," *Journal of AOAC International*, vol. 91, pp. 1227–1236, 2008.

[45] S. Lobasso, P. Lopalco, R. Angelini et al., "Coupled TLC and MALDI-TOF/MS analyses of the lipid extract of the hyperthermophilic archaeon pyrococcus furiosus," *Archaea*, vol. 2012, Article ID 957852, 10 pages, 2012.

[46] B. Fuchs, "Analysis of phospolipids and glycolipids by thin-layer chromatography-matrix-assisted laser desorption and ionization mass spectrometry," *Journal of Chromatography A*, vol. 1259, pp. 62–73, 2012.

[47] B. Fuchs, J. Schiller, R. Süß et al., "Analysis of stem cell lipids by offline HPTLC-MALDI-TOF MS," *Analytical and Bioanalytical Chemistry*, vol. 392, no. 5, pp. 849–860, 2008.

[48] B. Fuchs, J. Schiller, R. Süß, M. Schürenberg, and D. Suckau, "A direct and simple method of coupling matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS) to thin-layer chromatography (TLC) for the analysis of phospholipids from egg yolk," *Analytical and Bioanalytical Chemistry*, vol. 389, no. 3, pp. 827–834, 2007.

[49] G. Dobson, W. W. Christie, and B. Nikolova-Damyanova, "Silver ion chromatography of lipids and fatty acids," *Journal of Chromatography B: Biomedical Applications*, vol. 671, no. 1-2, pp. 197–222, 1995.

[50] B. Nikolova-Damyanova, "Silver ion chromatography and lipids," in *Advances in Lipid Methodology—One*, W. W. Christie, Ed., pp. 181–237, The Oily Press, Ayr, UK, 1992.

[51] B. Nikolova-Damyanova and S. Momchilova, "Silver ion thin-layer chromatography of fatty acids. A survey," *Journal of Liquid Chromatography and Related Technologies*, vol. 24, no. 10, pp. 1447–1466, 2001.

[52] B. Nikolova-Damyanova, W. W. Christie, and B. Herslöf, "Mechanistic aspects of fatty acid retention in silver ion chromatography," *Journal of Chromatography A*, vol. 749, no. 1-2, pp. 47–54, 1996.

[53] S. Momchilova and B. Nikolova-Damyanova, "Stationary phases for silver ion chromatography of lipids: preparation and properties," *Journal of Separation Science*, vol. 26, no. 3-4, pp. 261–270, 2003.

[54] D. Nikolova, D. Antonova, I. Marekov, and B. Nikolova-Damyanova, "Bis-methylene-interrupted octadecadienoic fatty acids in Bulgarian bovine butter fats," *European Journal of Lipid Science and Technology*, vol. 108, no. 3, pp. 212–217, 2006.

[55] R. Wilson and J. R. Sargent, "High-resolution separation of polyunsaturated fatty acids by argentation thin-layer chromatography," *Journal of Chromatography A*, vol. 623, no. 2, pp. 403–407, 1992.

[56] R. Wilson and J. R. Sargent, "Chain separation of monounsaturated fatty acid methyl esters by argentation thin-layer chromatography," *Journal of Chromatography A*, vol. 905, no. 1-2, pp. 251–257, 2001.

[57] D. S. Lin, W. E. Connor, D. P. Wolf, M. Neuringer, and D. L. Hachey, "Unique lipids of primate spermatozoa: desmosterol and docosahexaenoic acid," *The Journal of Lipid Research*, vol. 34, no. 3, pp. 491–499, 1993.

[58] E. H. Belarbi, E. Molina, and Y. Chisti, "A process for high yield and scaleable recovery of high purity eicosapentaenoic acid esters from microalgae and fish oil," *Enzyme and Microbial Technology*, vol. 26, no. 7, pp. 516–529, 2000.

[59] J. T. Dillon, J. C. Aponte, Y.-J. Tsai, and Y. Huang, "Thin layer chromatography in the separation of unsaturated organic compounds using silver-thiolate chromatographic material," *Journal of Chromatography A*, vol. 1251, pp. 240–243, 2012.

[60] J. M. Sowa and P. V. Subbaiah, "Variable recoveries of fatty acids following the separation of lipids on commercial silica gel TLC plates: selective loss of unsaturated fatty acids on certain brands of plates," *Journal of Chromatography B*, vol. 813, no. 1-2, pp. 159–166, 2004.

[61] A. M. Shahin, M. K. McGuire, M. A. McGuire, K. L. Ritzenthaler, and T. D. Shultz, "Determination of c9 ,t11 -CLA in major human plasma lipid classes using a combination of methylating methodologies," *Lipids*, vol. 38, no. 7, pp. 793–800, 2003.

[62] J. K. G. Kramer, C. Cruz-Hernandez, Z. Deng, J. Zhou, G. Jahreis, and M. E. R. Dugan, "Analysis of conjugated linoleic acid and trans 18:1 isomers in synthetic and animal products," *The American Journal of Clinical Nutrition*, vol. 79, no. 6, pp. 1137S–1145S, 2004.

[63] V. Fournier, P. Juanéda, F. Destaillats et al., "Analysis of eicosapentaenoic and docosahexaenoic acid geometrical isomers formed during fish oil deodorization," *Journal of Chromatography A*, vol. 1129, no. 1, pp. 21–28, 2006.

[64] E. Bailey-Hall, E. B. Nelson, and A. S. Ryan, "Validation of a rapid measure of blood PUFA levels in humans," *Lipids*, vol. 43, no. 2, pp. 181–186, 2008.

[65] J. Flieger, H. Szumiło, and K. Giełzak-Koćwin, "Optimizing modification conditions of silica gel with metal salts," *Journal of Liquid Chromatography and Related Technologies*, vol. 22, no. 19, pp. 2879–2894, 1999.

[66] D. Allan and S. Cockcroft, "A modified procedure for thin layer chromatography of phospholipids," *Journal of Lipid Research*, vol. 23, no. 9, pp. 1373–1374, 1982.

[67] W.-Q. Wang and A. Gustafson, "One-dimensional thin-layer chromatographic separation of phospholipids and lysophospholipids from tissue lipid extracts," *Journal of Chromatography - Biomedical Applications*, vol. 581, no. 1, pp. 139–142, 1992.

[68] J. L. Beneytout, D. Greuet, and M. T. Rigaud, "Separation of arachidonic acid metabolites by thin-layer chromatography using new silicone-bonded plates," *Journal of High Resolution Chromatography*, vol. 7, no. 9, pp. 538–539, 1984.

[69] J. Pikul and F. A. Kummerow, "Thiobarbituric acid reactive substance formation as affected by distribution of polyenoic fatty acids in individual phospholipids," *Journal of Agricultural and Food Chemistry*, vol. 39, no. 3, pp. 451–457, 1991.

[70] M. J. Martinez-Lorenzo, I. Marzo, J. Naval, and A. Pineiro, "Self-staining of polyunsaturated fatty acids in argentation thin-layer chromatography," *Analytical Biochemistry*, vol. 220, no. 1, pp. 210–212, 1994.

[71] B. Fuchs and J. Schiller, "MALDI-TOF MS analysis of lipids from cells, tissues and body fluids," in *Lipids in Health and Disease*, P. J. Quinn and X. Wang, Eds., vol. 49 of *Subcellular Biochemistry*, pp. 541–565, Springer, Berlin, Germany, 2008.

[72] B. Fuchs, K. Arnold, and J. Schiller, "Mass spectrometry of biological molecules," in *Encyclopedia of Analytical Chemistry*, R. A. Meyers, Ed., pp. 1–39, John Wiley & Sons, Chichester, UK, 2008.

[73] V. J. Sinanoglou, I. F. Strati, S. M. Bratakos, C. Proestos, P. Zoumpoulakis, and S. Miniadis-Meimaroglou, "On the combined application of Iatroscan TLC-FID and GC-FID to identify total, neutral, and polar lipids and their fatty acids extracted from foods," *ISRN Chromatography*, vol. 2013, Article ID 859024, 8 pages, 2013.

[74] A. Zabala, M. P. Portillo, M. T. Macarulla, V. M. Rodríguez, and A. Fernández-Quintela, "Effects of cis-9,trans-11 and trans-10,cis-12 CLA isomers on liver and adipose tissue fatty acid profile in hamsters," *Lipids*, vol. 41, no. 11, pp. 993–1001, 2006.

[75] I. Marekov, S. Panayotova, and R. Tarandjiiska, "Fatty acid composition and seasonal variation of trans fatty acid content in Bulgarian butter fats by silver ion TLC and GC," *Journal of Liquid Chromatography and Related Technologies*, vol. 32, no. 9, pp. 1183–1195, 2009.

[76] C. Cruz-Hernandez, Z. Deng, J. Zhou et al., "Methods for analysis of conjugated linoleic acids and trans-18:1 isomers in

dairy fats by using a combination of gas chromatography, silver-ion thin-layer chromatography/gas chromatography, and silver-ion liquid chromatography," *Journal of AOAC International*, vol. 87, no. 2, pp. 545–562, 2004.

[77] S. D. Krämer, J. A. Hurley, N. J. Abbott, and D. J. Begley, "Lipids in blood-brain barrier models in vitro I: thin-layer chromatography and high-performance liquid chromatography for the analysis of lipid classes and long-chain polyunsaturated fatty acids," *In Vitro Cellular & Developmental Biology—Animal*, vol. 38, no. 10, pp. 557–565, 2002.

[78] A. Laffargue, A. de Kochko, and S. Dussert, "Development of solid-phase extraction and methylation procedures to analyse free fatty acids in lipid-rich seeds," *Plant Physiology and Biochemistry*, vol. 45, no. 3-4, pp. 250–257, 2007.

[79] G. Chinnasamy, P. J. Davis, and A. K. Bal, "Seasonal changes in oleosomic lipids and fatty acids of perennial root nodules of beach pea," *Journal of Plant Physiology*, vol. 160, no. 4, pp. 355–365, 2003.

[80] A. Kotani, T. Fuse, and F. Kusu, "Determination of plasma free fatty acids by high-performance liquid chromatography with electrochemical detection," *Analytical Biochemistry*, vol. 284, no. 1, pp. 65–69, 2000.

[81] T. Řezanka, L. Nedbalová, and K. Sigler, "Odd-numbered very-long-chain polyunsaturated fatty acids from the dinoflagellate *Amphidinium carterae* identified by atmospheric pressure chemical ionization liquid chromatography-mass spectrometry," *Phytochemistry*, vol. 69, no. 16, pp. 2849–2855, 2008.

[82] M. P. Mansour, "Reversed-phase high-performance liquid chromatography purification of methyl esters of C16-C28 polyunsaturated fatty acids in microalgae, including octacosaoctaenoic acid [28:8(n-3)]," *Journal of Chromatography A*, vol. 1097, no. 1-2, pp. 54–58, 2005.

[83] S. Momchilova and B. Nikolova-Damyanova, "Silver ion high performance liquid chromatography of polynuclear aromatic derivatives of positionally isomeric octadecenoic fatty acids," *Journal of Liquid Chromatography and Related Technologies*, vol. 23, no. 9, pp. 1319–1330, 2000.

[84] J. J. Han, Y. Iwasaki, and T. Yamane, "Use of isopropanol as a modifier in a hexane-acetonitrile based mobile phase for the silver ion HPLC. Separation of positional isomers of triacylglycerols containing long chain polyunsaturated fatty acids," *Journal of High Resolution Chromatography*, vol. 22, pp. 357–361, 1999.

[85] F. Destaillats, J.-L. Sébédio, O. Berdeaux, P. Juanéda, and P. Angers, "Gas chromatography-mass spectrometry determination of metabolites of conjugated cis-9,trans-11,cis-15 18:3 fatty acid," *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, vol. 820, no. 1, pp. 15–22, 2005.

[86] M. P. Mansour, J. K. Volkman, D. G. Holdsworth, A. E. Jackson, and S. I. Blackburn, "Very-long-chain (C28) highly unsaturated fatty acids in marine dinoflagellates," *Phytochemistry*, vol. 50, no. 4, pp. 541–548, 1999.

[87] T. Řezanka and V. M. Dembitsky, "Very long chain polyunsaturated fatty acids in crustacea of the order *Bathynellacea*," *Biochemical Systematics and Ecology*, vol. 27, no. 6, pp. 551–558, 1999.

[88] L. Akoto, R. Pel, H. Irth, U. A. T. Brinkman, and R. J. J. Vreuls, "Automated GC-MS analysis of raw biological samples: application to fatty acid profiling of aquatic micro-organisms," *Journal of Analytical and Applied Pyrolysis*, vol. 73, no. 1, pp. 69–75, 2005.

[89] C. Böcking, W. A. Nockher, M. Schreiner, H. Renz, and P. I. Pfefferle, "Development and validation of a combined method for the biomonitoring of omega-3/-6 fatty acids and conjugated linoleic acids in different matrices from human and nutritional sources," *Clinical Chemistry and Laboratory Medicine*, vol. 48, no. 12, pp. 1757–1763, 2010.

[90] C. Tyburczy, P. Delmonte, A. R. Fardin-Kia, M. M. Mossoba, J. K. G. Kramer, and J. I. Rader, "Profile of trans fatty acids (FAs) including trans polyunsaturated fas in representative fast food samples," *Journal of Agricultural and Food Chemistry*, vol. 60, no. 18, pp. 4567–4577, 2012.

[91] M. M. M. Hassanein and A. G. Abedel-Razek, "Chromatographic quantitation of some bioactive minor components in oils of wheat germ and grape seeds produced as by-products," *Journal of Oleo Science*, vol. 58, no. 5, pp. 227–233, 2009.

[92] A. Stołyhwo, I. Kołodziejska, and Z. E. Sikorski, "Long chain polyunsaturated fatty acids in smoked Atlantic mackerel and Baltic sprats," *Food Chemistry*, vol. 94, no. 4, pp. 589–595, 2006.

[93] E. S. Lima and D. S. P. Abdalla, "High-performance liquid chromatography of fatty acids in biological samples," *Analytica Chimica Acta*, vol. 465, no. 1-2, pp. 81–91, 2002.

[94] M. S. Rao, K. Hidajat, and C. B. Ching, "Reversed-phase HPLC: the separation method for the characterization and purification of long chain polyunsaturated fatty acids—a review," *Journal of Chromatographic Science*, vol. 33, no. 1, pp. 9–21, 1995.

[95] A. Mehta, A. M. Oeser, and M. G. Carlson, "Rapid quantitation of free fatty acids in human plasma by high-performance liquid chromatography," *Journal of Chromatography B: Biomedical Applications*, vol. 719, no. 1-2, pp. 9–23, 1998.

[96] T. Rezanka and J. Votruba, "Chromatography of very long-chain fatty acids from animal and plant kingdoms," *Analytica Chimica Acta*, vol. 465, no. 1-2, pp. 273–297, 2002.

[97] W. Kolanowski, D. Jaworska, and J. Weissbrodt, "Importance of instrumental and sensory analysis in the assessment of oxidative deterioration of omega-3 long-chain polyunsaturated fatty acid-rich foods," *Journal of the Science of Food and Agriculture*, vol. 87, no. 2, pp. 181–191, 2007.

[98] G. A. Haward and A. J. P. Martin, "The separation of the C12-C18 fatty acids by reversed-phase partition chromatography," *Journal of Biochemistry*, vol. 46, pp. 532–538, 1950.

[99] J. M. Rosenfeld, "Application of analytical derivatizations to the quantitative and qualitative determination of fatty acids," *Analytica Chimica Acta*, vol. 465, no. 1-2, pp. 93–100, 2002.

[100] M. I. Aveldano, M. VanRollins, and L. A. Horrocks, "Separation and quantitation of free fatty acids and fatty acid methyl esters by reverse phase high pressure liquid chromatography," *Journal of Lipid Research*, vol. 24, no. 1, pp. 83–93, 1983.

[101] F.-H. Wang, X.-J. Xiong, X.-F. Guo, H. Wang, and H.-S. Zhang, "Determination of fatty acids in bio-samples based on the pre-column fluorescence derivatization with 1,3,5,7-tetramethyl-8-butyrethylenediamine-difluoroboradiaza-s-indacene by high performance liquid chromatography," *Journal of Chromatography A*, vol. 1291, pp. 84–91, 2013.

[102] T. Řezanka and K. Sigler, "Identification of very long chain fatty acids from sugar cane wax by atmospheric pressure chemical ionization liquid chromatography-mass spectroscopy," *Phytochemistry*, vol. 67, no. 9, pp. 916–923, 2006.

[103] J. Sajiki and J. Yonekubo, "Determination of free polyunsaturated fatty acids and their oxidative metabolites by high-performance liquid chromatography (HPLC) and mass spectrometry (MS)," *Analytica Chimica Acta*, vol. 465, no. 1-2, pp. 417–426, 2002.

[104] T. Řezanka, L. Nedbalová, and K. Sigler, "Identification of very-long-chain polyunsaturated fatty acids from *Amphidinium carterae* by atmospheric pressure chemical ionization liquid chromatography-mass spectroscopy," *Phytochemistry*, vol. 69, no. 12, pp. 2391–2399, 2008.

[105] T. Řezanka, O. Schreiberová, T. Krulikovská, J. Masák, and K. Sigler, "RP-HPLC/MS-APCI analysis of odd-chain TAGs from *Rhodococcus erythropolis* including some regioisomers," *Chemistry and Physics of Lipids*, vol. 163, no. 4-5, pp. 373–380, 2010.

[106] T. Řezanka, "Identification of very long chain fatty acids by atmospheric pressure chemical ionization liquid chromatography-mass spectroscopy from green alga *Chlorella kessleri*," *Journal of Separation Science*, vol. 25, no. 18, pp. 1332–1336, 2002.

[107] T. Řezanka and K. Sigler, "Identification of very long chain unsaturated fatty acids from Ximenia oil by atmospheric pressure chemical ionization liquid chromatography-mass spectroscopy," *Phytochemistry*, vol. 68, no. 6, pp. 925–934, 2007.

[108] T. Řezanka, "Analysis of very long chain polyunsaturated fatty acids using high-performance liquid chromatography—atmospheric pressure chemical ionization mass spectrometry," *Biochemical Systematics and Ecology*, vol. 28, no. 9, pp. 847–856, 2000.

[109] T. Řezanka and K. Sigler, "Odd-numbered very-long-chain fatty acids from the microbial, animal and plant kingdoms," *Progress in Lipid Research*, vol. 48, no. 3-4, pp. 206–238, 2009.

[110] M. Fer, S. Goulitquer, Y. Dréano, F. Berthou, L. Corcos, and Y. Amet, "Determination of polyunsatured fatty acid monoepoxides by high performance liquid chromatography-mass spectrometry," *Journal of Chromatography A*, vol. 1115, no. 1-2, pp. 1–7, 2006.

[111] M. Lísa, M. Holčapek, T. Řezanka, and N. Kabátová, "High-performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry and gas chromatography-flame ionization detection characterization of Δ5-polyenoic fatty acids in triacylglycerols from conifer seed oils," *Journal of Chromatography A*, vol. 1146, no. 1, pp. 67–77, 2007.

[112] J. I. Teng and N. M. M. Gowda, "Analysis of n-3 fatty acids in fish oils by high-performance liquid chromatography," *Chromatographia*, vol. 35, no. 9-12, pp. 627–630, 1993.

[113] R. W. Browne and D. Armstrong, "HPLC analysis of lipid-derived polyunsaturated fatty acid peroxidation products in oxidatively modified human plasma," *Clinical Chemistry*, vol. 46, no. 6, pp. 829–836, 2000.

[114] J.-T. Lin, T. A. McKeon, and A. E. Stafford, "Gradient reversed-phase high-performance liquid chromatography of saturated, unsaturated and oxygenated free fatty acids and their methyl esters," *Journal of Chromatography A*, vol. 699, no. 1-2, pp. 85–91, 1995.

[115] M. Yamaguchi, R. Matsunaga, K. Fukuda, M. Nakamura, and Y. Ohkura, "Highly sensitive determination of free polyunsaturated, long-chain fatty acids in human serum by high-performance liquid chromatography with fluorescence detection," *Analytical Biochemistry*, vol. 155, no. 2, pp. 256–261, 1986.

[116] K. Nishimura, T. Suzuki, S. Momchilova, K. Miyashita, E. Katsura, and Y. Itabashi, "Analysis of conjugated linoleic acids as 9-anthrylmethyl esters by reversed-phase high-performance liquid chromatography with fluorescence detection," *Journal of Chromatographic Science*, vol. 43, no. 9, pp. 494–499, 2005.

[117] N. Noda, K. Umebayashi, T. Nakatani, K. Miyahara, and K. Ishiyama, "Isolation and characterization of some hydroxy fatty and phosphoric acid esters of 10-hydroxy-2-decenoic acid from the royal jelly of honeybees (*Apis mellifera*)," *Lipids*, vol. 40, no. 8, pp. 833–838, 2005.

[118] B. Nikolova-Damyanova and S. Momchilova, "Silver ion HPLC for the analysis of positionally isomeric fatty acids," *Journal of Liquid Chromatography & Related Technologies*, vol. 25, no. 13–15, pp. 1947–1965, 2002.

[119] N. M. Aini, M. M. Yusoff, and H. A. Azhari, "Chromatographic methods to analyze geometrical and positional isomers of fatty acids. A review," in *Proceedings of the National Conference on Postgraduate Research (NCON-PGR '09)*, M. M. Noor, M. M. Rahman, and K. Kadirgama, Eds., UMP Conference Hall, pp. 165–175, Pahang, Malaysia, 2009.

[120] S. Momchilova, B. Nikolova-Damyanova, and W. W. Christie, "Silver ion high-performance liquid chromatography of isomeric cis- and trans-octadecenoic acids. Effect of the ester moiety and mobile phase composition," *Journal of Chromatography A*, vol. 793, no. 2, pp. 275–282, 1998.

[121] J. L. Guil-Guerrero, P. Campra-Madrid, and R. Navarro-Juárez, "Isolation of some PUFA from edible oils by argentated silica gel chromatography," *Grasas y Aceites*, vol. 54, no. 2, pp. 116–121, 2003.

[122] C. Sun, Y. Y. Zhao, and J. M. Curtis, "The direct determination of double bond positions in lipid mixtures by liquid chromatography/in-line ozonolysis/mass spectrometry," *Analytica Chimica Acta*, vol. 762, pp. 68–75, 2013.

[123] S. Casal and B. Oliveira, "Fatty acids: GC analysis," in *Encyclopedia of Chromatography*, J. Cazes, Ed., pp. 833–845, Taylor & Francis, New York, NY, USA, 3rd edition, 2010.

[124] T. Seppänen-Laakso, I. Laakso, and R. Hiltunen, "Analysis of fatty acids by gas chromatography, and its relevance to research on health and nutrition," *Analytica Chimica Acta*, vol. 465, no. 1-2, pp. 39–62, 2002.

[125] R. G. Ackman, "The gas chromatograph in practical analyses of common and uncommon fatty acids for the 21st century," *Analytica Chimica Acta*, vol. 465, no. 1-2, pp. 175–192, 2002.

[126] M. W. Muriuki, G. G. Dumancas, N. Purdie, and L. Reilly, "Quantification of total ω-6 and ω-3 fatty acids and ω-6/ω-3 ratio in human serum using GC-MS," *LC-GC North America*, vol. 29, no. 1, pp. 60–65, 2011.

[127] H. Kawashima and M. Ohnishi, "Identification of minor fatty acids and various nonmethylene-interrupted diene isomers in mantle, muscle, and viscera of the marine bivalve *Megangulus zyonoensis*," *Lipids*, vol. 39, no. 3, pp. 265–271, 2004.

[128] N. Tsevegsuren, K. Fujimoto, W. W. Christie, and Y. Endo, "Occurrence of a novel cis,cis,cis-octadeca-3,9,12-trienoic (Z,Z,Z-octadeca-3,9,12-trienoic) acid in Chrysanthemum (Tanacetum) zawadskii Herb. (Compositae) seed oil," *Lipids*, vol. 38, no. 5, pp. 573–578, 2003.

[129] A. L. Michaud, M. P. Yurawecz, P. Delmonte, B. A. Corl, D. E. Bauman, and J. T. Brenna, "Identification and characterization of conjugated fatty acid methyl esters of mixed double bond geometry by acetonitrile chemical ionization tandem mass spectrometry," *Analytical Chemistry*, vol. 75, no. 18, pp. 4925–4930, 2003.

[130] J. Ecker, M. Scherer, G. Schmitz, and G. Liebisch, "A rapid GC-MS method for quantification of positional and geometric isomers of fatty acid methyl esters," *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, vol. 897, pp. 98–104, 2012.

[131] F. Destaillats, P.-A. Golay, F. Joffre et al., "Comparison of available analytical methods to measure trans-octadecenoic

acid isomeric profile and content by gas-liquid chromatography in milk fat," *Journal of Chromatography A*, vol. 1145, no. 1-2, pp. 222–228, 2007.

[132] A. Liu, R. Terry, Y. Lin, K. Nelson, and P. S. Bernstein, "Comprehensive and sensitive quantification of long-chain and very long-chain polyunsaturated fatty acids in small samples of human and mouse retina," *Journal of Chromatography A*, vol. 1307, pp. 191–200, 2013.

[133] M. Schreiner, "Quantification of long chain polyunsaturated fatty acids by gas chromatography: evaluation of factors affecting accuracy," *Journal of Chromatography A*, vol. 1095, no. 1-2, pp. 126–130, 2005.

[134] V. Bláha, D. Solichová, D. Černohorský, M. Brátová, P. Vyroubal, and Z. Zadák, "Bioanalysis of PUFA metabolism and lipid peroxidation in coronary atherosclerosis," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 22, no. 3, pp. 563–572, 2000.

[135] E. Tvrzická, M. Vecka, B. Staňková, and A. Žák, "Analysis of fatty acids in plasma lipoproteins by gas chromatography-flame ionization detection: quantitative aspects," *Analytica Chimica Acta*, vol. 465, no. 1-2, pp. 337–350, 2002.

[136] Z. Xu, K. Harvey, T. Pavlina, G. Dutot, G. Zaloga, and R. Siddiqui, "An improved method for determining medium- and long-chain FAMEs using gas chromatography," *Lipids*, vol. 45, no. 2, pp. 199–208, 2010.

[137] J. K. G. Kramer, C. B. Blackadar, and J. Zhou, "Evaluation of two GC columns (60-m SUPELCOWAX 10 and 100-m CP Sil 88) for analysis of milkfat with emphasis on CLA, 18:1, 18:2 and 18:3 isomers, and short- and long-chain FA," *Lipids*, vol. 37, no. 8, pp. 823–835, 2002.

[138] P. Delmonte and J. I. Rader, "Evaluation of gas chromatographic methods for the determination of trans fat," *Analytical and Bioanalytical Chemistry*, vol. 389, no. 1, pp. 77–85, 2007.

[139] A. Timmins, E. J. MacPherson, and R. G. Ackman, "Direct use of methyl tricosanoate as an internal standard and overcoming a potential error in the quantitation of the omega-3 long-chain polyunsaturated fatty acids of marine oils as ethyl esters," *Food Chemistry*, vol. 70, no. 3, pp. 425–426, 2000.

[140] P. A. Golay, F. Giuffrida, F. Dionisi, and F. Destaillats, "Streamlined methods for the resolution and quantification of fatty acids including trans fatty acid isomers in food products by gas chromatography," *Journal of AOAC International*, vol. 92, no. 5, pp. 1301–1309, 2009.

[141] C. Ragonese, P. Q. Tranchida, D. Sciarrone, and L. Mondello, "Conventional and fast gas chromatography analysis of biodiesel blends using an ionic liquid stationary phase," *Journal of Chromatography A*, vol. 1216, no. 51, pp. 8992–8997, 2009.

[142] P. Delmonte, Q. Hu, A.-R. F. Kia, and J. I. Rader, "Preparation, chromatographic separation and relative retention times of *cis/trans* heptadecaenoic (17:1) fatty acids," *Journal of Chromatography A*, vol. 1214, no. 1-2, pp. 30–36, 2008.

[143] Q. Gu, F. David, F. Lynen et al., "Evaluation of ionic liquid stationary phases for one dimensional gas chromatography-mass spectrometry and comprehensive two dimensional gas chromatographic analyses of fatty acids in marine biota," *Journal of Chromatography A*, vol. 1218, no. 20, pp. 3056–3063, 2011.

[144] P. Manzano, E. Arnáiz, J. C. Diego et al., "Comprehensive two-dimensional gas chromatography with capillary flow modulation to separate FAME isomers," *Journal of Chromatography A*, vol. 1218, no. 30, pp. 4952–4959, 2011.

[145] W. Meier-Augenstein, "Stable isotope analysis of fatty acids by gas chromatography-isotope ratio mass spectrometry," *Analytica Chimica Acta*, vol. 465, no. 1-2, pp. 63–79, 2002.

[146] H. Azzian, J. K. G. Kramer, A. R. Kamalian et al., "Quantification of trans fatty acids in food products by GC, ATR-FTIR and FT-NIR methods," *Lipid Technology*, vol. 16, pp. 229–231, 2004.

[147] L. T. Taylor, "Supercritical fluid chromatography for the 21st century," *The Journal of Supercritical Fluids*, vol. 47, no. 3, pp. 566–573, 2009.

[148] F. J. Señoráns and E. Ibañez, "Analysis of fatty acids in foods by supercritical fluid chromatography," *Analytica Chimica Acta*, vol. 465, no. 1-2, pp. 131–144, 2002.

[149] J. L. Bernal, M. T. Martin, and L. Toribio, "Supercritical fluid chromatography in food analysis," *Journal of Chromatography A*, vol. 1313, pp. 24–36, 2013.

[150] G. Pettinello, A. Bertucco, P. Pallado, and A. Stassi, "Production of EPA enriched mixtures by supercritical fluid chromatography: from the laboratory scale to the pilot plant," *Journal of Supercritical Fluids*, vol. 19, no. 1, pp. 51–60, 2000.

[151] T. Bamba, N. Shimonishi, A. Matsubara et al., "High throughput and exhaustive analysis of diverse lipids by using supercritical fluid chromatography-mass spectrometry for metabolomics," *Journal of Bioscience and Bioengineering*, vol. 105, no. 5, pp. 460–469, 2008.

Hindawi Publishing Corporation
Journal of Chemistry
Volume 2015, Article ID 853789, 16 pages
http://dx.doi.org/10.1155/2015/853789

Review Article

Status of the Reactive Extraction as a Method of Separation

Dipaloy Datta,[1] Sushil Kumar,[2] and Hasan Uslu[3]

[1]*Chemical Engineering Department, Thapar University, Patiala 147004, India*
[2]*Department of Chemical Engineering, Motilal Nehru National Institute of Technology (MNNIT), Allahabad, Uttar Pradesh 211004, India*
[3]*Chemical Engineering Department, Engineering and Architecture Faculty, Beykent University, Ayazağa, 34437 İstanbul, Turkey*

Correspondence should be addressed to Hasan Uslu; hasanuslu@gmail.com

Received 7 August 2014; Accepted 20 September 2014

Academic Editor: Saeid Azizian

Copyright © 2015 Dipaloy Datta et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The prospective function of a novel energy efficient fermentation technology has been getting great attention in the past fifty years due to the quick raise in petroleum costs. Fermentation chemicals are still limited in the modern market in huge part because of trouble in recovery of carboxylic acids. Therefore, it is needed considerable development in the current recovery technology. Carboxylic acids have been used as the majority of fermentation chemicals. This paper presents a state-of-the-art review on the reactive extraction of carboxylic acids from fermentation broths. This paper principally focuses on reactive extraction that is found to be a capable option to the proper recovery methods.

1. Introduction

Liquid-liquid extraction (LLE) is a process in which a particular solute is removed from a liquid phase (feed phase) by another liquid phase (solvent or extract phase). Application of extraction in life science started in way back to about 3500 BC to recover products from various natural resources. At a Sumerian text dated 2100 BC, production of perfumes, pharmaceutical oils, and waxes was documented. In medieval ages extraction was performed with ethanol and was applied in the field of hydrometallurgy by making use of mineral acids [1]. With the developments in thermodynamics, particularly the distribution law by Nernst in 1891 and design of an apparatus for extraction, significant improvements were accomplished in the late 19th century. However, it was not until the early 1930s when the first large-scale LLE process was in operation. Lazar Edeleanu (a Romanian Chemist: 1861–1941) removed aromatic and sulphur compounds from liquid kerosene by the LLE process using liquid sulphur dioxide as a solvent at temperature as low as −6 to −12°C. This yielded clean kerosene suitable to be used as a fuel for residential lighting. Nowadays, besides the extraction of almost all metals in mining industries and environmental applications, extractants are widely used in the extraction of organic and inorganic acids, organic chemistry intermediates, and pharmaceuticals for the purposes of separation, purification, or enrichment [2] of the product.

The main difference between reactive extraction and solvent extraction is the reaction between the extractant and the solute in the organic phase. Aliphatic amines and phosphoric solvents are proposed as effective extractants by earlier researchers [3]. While extractants play the major role in the reaction, diluents also have a significant effect on the level of extraction. Nonaromatic, water immiscible, and polar solvents with intermediate molecular weights and high boiling points are commonly preferred for the extraction to have high distribution and selectivity [4]. The solvents (diluents) control the physical properties (viscosity, density, surface tension, etc.) of the solvent phase and also affect the stability of the complex structure formed between the solute and the extractant.

The reactive extraction is found to be a promising method for the recovery of the carboxylic acids from a dilute fermentation broth [5–10]. This separation method has advantages such as the following:

(i) effective at high concentration of substrate in the extractive fermentation,

(ii) the acid can be reextracted and the solvent can be reused,

(iii) better control of pH in the bioreactor,

(iv) better recovery of acid with higher product purity,

(v) reduction of downstream processing load and recovery cost,

(vi) reactants are relatively immiscible,

(vii) product(s) undergoes subsequent undesired reactions in reaction phase,

(viii) reaction products to be separated are immiscible with the reaction phase,

(ix) phase equilibrium can be positively influenced,

(x) heat transfer is to be improved during the reaction,

(xi) product-catalyst separation can be affected by a liquid-liquid separation.

Kertes and King [3] categorize the extractants as three major types:

(i) carbon bonded oxygen bearing extractants,

(ii) phosphorus bonded oxygen bearing extractants,

(iii) high molecular weight aliphatic amines.

The first two categories are nonreactive in nature and extract the acid molecules by solvation. The distinction between the first two categories is based on the strength of the solvation bonds and the specificity of solvation. The coordinate bonds between carbon bonded oxygen donor extractant and the acid are too weak for a specific solvation but it is significantly more for phosphorus bonded oxygen bearing extractants. The extractants in the second category make the solvation process more specific and the number of solvating molecules per extracted acid molecule can be accessible experimentally. The aliphatic amines in the third category can react with the carboxylic acid molecule and form acid-amine complexes by proton transfer or by ion pair formation. This causes a significant increase in the distribution coefficient of the carboxylic acid [11]. Among aliphatic amines, primary alkyl amines are observed to be excessively soluble in water at room temperature while secondary amines form a gel phase (third phase) at the interface which create difficulty in phase separation [3]. The extractability of tertiary amines is found to be more than that of the primary and secondary amines [5]. Aliphatic tertiary amines having more than six carbon atoms per chain are found to be effective extractants for the recovery of carboxylic acids [3].

Although a tertiary amine has good extractability, it must always be used with a diluent due to its viscous and corrosive nature. Further, the stability of the formed acid-amine complexes in the reactive extraction is affected by the basicity of the amine which can be manipulated by using different types of diluents. Moreover, use of a diluent controls the physical properties such as density, viscosity, and surface tension of the organic phase [12].

Diluents can be broadly divided into two groups: (i) active diluents and (ii) inactive diluents. Generally, the active diluents are polar in nature due to the presence of functional groups. They are good solvating media for an ion-pair such as an acid-amine complex [13]. The category includes chlorinated hydrocarbon, ketone, alcohol, and halogenated aromatic solvents. Inactive diluents being nonpolar provide very low distribution of the acid and poor solvation of the polar complexes. Alkanes, benzene, alkyl substituted aromatics, and so forth fall in this category. These diluents limit the formation of the third phase at higher concentrations of acid in the organic phase and are useful in the stripping of acid. The equilibrium curve can be shifted towards the aqueous phase by increasing the concentration of the inert diluent in the mixture of diluents [14].

Reactive extraction represents a reaction between the acid (solute) and extractant molecule at the interface of aqueous and organic phase where transfers of acid molecules take place by the diffusion and solubilization mechanism. Reactive extraction strongly depends on various parameters such as aqueous phase composition, organic phase composition, types of complexes (1 : 1, 2 : 1, etc.) formed, properties of the solvent (extractant and diluent), type of solvent, temperature, and pH [15]. The purpose should be achieving a high distribution coefficient with higher selectivity. This can be realized by utilizing an appropriate organic phase at optimum conditions.

There are two stages in extractive separation (Figure 1). The first is the extraction of the solute to produce a solute-extractant complex and a relatively solute free aqueous raffinate. The second step is necessary for stripping the solute from the organic complex to obtain amine free aqueous solute as a product and also for simultaneously regenerating the extractant recycled back to the extraction unit. In the second stage of reactive extraction, it is necessary to regenerate the organic phase. Tamada and King [16] have described two approaches for the regeneration of extractant-diluent system: (i) temperature swing regeneration and (ii) diluent swing regeneration. Yabannavar and Wang [17] have purified lactic acid from a loaded organic phase using sodium hydroxide (NaOH) and hydrochloric acid (HCl). Poole and King [18] have used an aqueous solution of low molecular weight amine for complete regeneration of organic phase.

Reactive extraction has many applications in chemical and pharmaceutical industries (intermediates, organic acids, vitamins...) or in hydrometallurgical and environmental science (heavy metals, inorganic acids...). The liquid ion exchangers are used to promote a certain reaction to accomplish very selective separations [19]. There are many liquid ion exchangers commercially available. They work basically with three different types of mechanisms: anion exchange, cation exchange, and solvation. It is practical to use ion-exchangers in a diluent which is immiscible with water since they are highly viscous or solid materials. The diluent makes the organic phase easier to handle [1].

There are a significant number of studies on reactive extraction of organic acids in the literature. These studies investigate various aspects of reactive extraction of organic acids: chemical interactions between acids and extractants, reaction mechanisms, type of extractants and diluents, effect of temperature and pH, effect of aqueous and organic phase

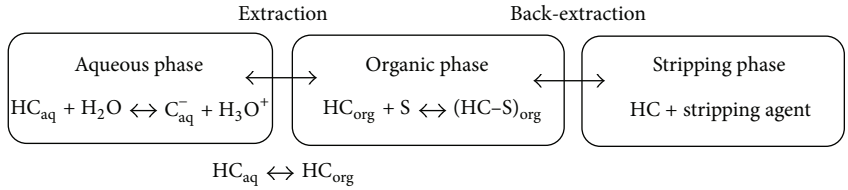


Figure 1: Schematic representation of the reactive extraction process (extraction and back-extraction).

compositions on extraction, effect of modifiers, extractive fermentation processes, kinetics of extraction, stripping the acid from the organic phase, and so forth.

2. Equilibrium Studies on Reactive Extraction of Carboxylic Acids

Several researchers [3, 8, 9, 13, 16, 21, 26, 31, 56–59] have studied theoretical and experimental aspects of the recovery of carboxylic acids from their aqueous solutions by reactive extraction. In their work, the investigators have examined the effects of various parameters on the distribution of the acid (solute) between the aqueous and organic phase. Some of these parameters are concentration and composition of both phases, types of the extractants and diluents, pH of the aqueous phase, temperature of the system, and toxicity of the solvent phase to the microorganism [3, 13, 57, 60, 61]. In addition to these experimental studies, the possible reaction mechanisms are also described and determined using different equilibrium and kinetic models [8, 9, 50, 52]. Some experimental results have showed synergistic and antagonistic effects on the extraction of the carboxylic acids when there is more than one acid in the aqueous phase or more than one extractant in the organic phase [21, 62, 63]. Some researchers have tried to recover the carboxylic acids from production medium by extractive fermentation and studied the process conditions affecting recovery during production [57, 60, 61, 64].

King and his group have performed the pioneering studies on the equilibrium of reactive extraction of carboxylic acids. Besides carboxylic acids, they have also studied the extraction of chlorinated hydrocarbons and aromatics [65], ethanol [66], ammonia [67], and low molecular weight aliphatic alcohols [3] from aqueous solutions. In one of their earlier work, Wardell and King [11] studied the extraction of acetic and formic acids from their aqueous solutions [11] using phosphoryl solvents (tributyl phosphate, dibutyl phosphonate, tributylphosphineoxide, and triphenylphosphineoxide) and tertiary amine extractants (tri-*n*-octylamine and tri-iso-octylamine) dissolving in different solvents. High distribution coefficients are achieved with phosphoryl compounds which act as a Lewis base (presence of the phosphoryl bond, P–O). The results indicated that, with the increase in the electronegativity, a decrease in the electron-donating ability and disappearance of Lewis basicity were observed. This study also showed the advantage of using long chain amines as extractants in the recovery of carboxylic acids. It was also observed that the extent of the extraction of acetic acid appeared to increase with an increase in the solubility parameter of the diluent besides the polarity.

At the later stage, Kertes and King [3], in 1986, published a research article in which the authors discussed the improvements in fermentation technology and its need of commercialization. They have reviewed 11 carboxylic acids (propionic, pyruvic, lactic, succinic, fumaric, maleic, itaconic, tartaric, citric, and isocitric) which are obtained by aerobic fermentation of glucose via the glycolytic pathway and glyoxylate bypass. The investigators pointed out that it is the undissociated part of a monocarboxylic acid which can only be extracted into carbon-bonded and phosphorus-bonded solvent. This revealed that the initial pH of the aqueous solution and the dissociation constant (pK_a) of the acid are two very important and influential parameters in the extraction of particular acid. Mass action law and Nernst distribution law are used in order to evaluate the data. The authors have considered the dimerization of acids in the organic phase and proposed a relation between dimerization constant and partition coefficient. The emphasis is given for the use of aliphatic tertiary amines compared to primary and secondary amines. In particular, monocarboxylic acids under comparable conditions are noted to be more easily extracted with an appropriate organic phase than di- or tri-carboxylic acids [3].

King and his coworkers [13, 16, 56] continued their studies on reactive extraction of carboxylic acids. In their first study [13], they have carried out the extraction equilibrium experiments of carboxylic acids (acetic, lactic, succinic, malonic, fumaric, and maleic) with different pK_a values. They have studied the effect of pK_a of the corresponding acid on the extent of extraction. It is found that the acid with higher pK_a value is extracted in more amounts. Furthermore, they have also searched for the effect of functional groups present in acid other than the primary carboxyl group on extraction. The reactive extraction is examined using Alamine 336 dissolved in various diluents (active diluents: 1-octanol, DCM, chloroform, MIBK, nitrobenzene; inert diluent: heptane). These diluents are selected with different chemical characteristics such as electron donating, electron accepting, polar and nonpolar in order to observe the effect of diluent-complex interactions on the equilibrium conditions and the possible formation of acid-amine complexes (1 : 1, 2 : 1). They have indicated that solubility of the acid-amine complex in the solvent phase is decreased in the following order: alcohol ≥ nitrobenzene ≥ proton donating halogenated hydrocarbon > ketone > halogenated aromatic > benzene > alkyl aromatic > aromatic hydrocarbon.

In their second study, Tamada and King [56] have investigated the chemical interactions between the components by using the results of mass action law analysis and the spectroscopic studies. Organic phase is analyzed by infrared spectroscopy to examine the stoichiometry of the acid-amine complex. They emphasize that there is an ion pair formation between the amine and first acid molecule and there is a hydrogen bond formation between the carboxyl of the second acid molecule and the carboxylate of the first in the formation of 2 : 1 complex of acid-amine.

In the last part of the study [16], this group has carried out the coextraction of water with the acids by Alamine 336 dissolved in various diluents and found that amine had no effect on the coextraction of water. Water coextraction was decreased in the order of 1-octanol > MIBK > nitrobenzene > methylene chloride > chloroform > heptane during the extraction of succinic acid. Coextraction of water for different acids is also compared and it is revealed that monocarboxylic acids carry less water than dicarboxylic acids. In this study, the effect of the temperature on the reactive extraction of carboxylic acids by Alamine 336 is performed. It is observed that the distribution coefficient decreased with the increase in the temperature of the system as with the formation of the complex, the system became more ordered and entropy decreased. Consequently, the amount of acid extracted decreased with the increase in temperature. Finally, King and his coworkers regenerated the extractant through back-extraction by two approaches: swing temperature and swing diluent methods.

The quaternary amines are first studied by Yang et al. [68]. They have investigated Alamine 336 and Aliquat 336, quaternary amine produced by the methylation of Alamine 336 and composed of a large organic cation with a chloride ion, dissolved in kerosene or 2-octanol to extract lactic, acetic, and propionic and butyric acids. It has been reported that while Alamine 336 can extract only the undissociated acid, Aliquat 336 can extract both dissociated and undissociated acids and always gives higher distribution coefficients than Alamine 336 at all pH values. It is also reported in this study that the extraction power of Alamine 336 is increased by the polar diluent, 2-octanol. On the other hand a diluent effect could not be detected on the extraction power of Aliquat 336.

Procházka et al. [69] have studied trialkylamine, a mixture of straight chain tertiary amine with 7–9 carbon atoms, dissolved in the mixture of 1-octanol and n-heptane to extract lactic, malic and citric acids. It is reported that all systems used in this study are sensitive to temperature and solvent composition. In another study, Juang et al. [21, 70] used a tertiary amine based extractant, tri-*n*-octylamine (TOA) dissolved in xylene to extract succinic and tartaric acids [21, 70]. They obtained thermodynamic data on extraction and compared extraction equilibria of succinic and tartaric acids with TOA dissolved in xylene. They have reported that the equilibrium distribution coefficient of the acids extracted with TOA increases with initial concentration of amine in the organic phase and of the acid in the aqueous phase. TOA has also been investigated by Poposka et al. [71] as an extractant dissolved in an iso decanol/n heptane mixture for extraction equilibria of citric acid. The data were obtained as a function of acid, amine and isodecanol concentrations at 298 K and an appropriate mathematical model was proposed.

Most of the studies on reactive extraction in the literature are focused on a single acid in the aqueous phase. However, Juang et al. [70] have reported that extraction characteristics are affected by the presence of more than one acid in the aqueous phase. Separation mechanisms of lactic and citric acids in the aqueous phase with TOA dissolved in xylene have been investigated via the supported liquid membrane technique. They have reported large synergistic effects compared to single acid systems for low initial concentration ratio of citric to lactic acid (α) and an antagonistic effect at $\alpha = 2$. The presence of the second acid has a positive effect on the transport of citric acid but a negative effect on that of the lactic acid. Transport rate of the acids also increases with TOA concentration and temperature. New synergistic extraction system for organic acids was developed by Matsumoto et al. [72]. The extraction equilibria were investigated for acetic, glycolic, propionic, lactic, succinic, fumaric, malic, and itaconic acids by tri-*n*-octylamine (TOA) and/or tri-n-butylphosphate (TBP) dissolved in hexane. It is reported that when a mixture of extractants are used a synergy is developed and a more effective extraction of all organic acids is achieved.

The above studies clearly show that the reaction between the extracted acid and the extractant present in the organic phase is dependent on the corresponding acid and the contents of the organic phase. To explore the possibilities of reactive extraction and its application and commercialization, further studies are carried out with different carboxylic acids using various extractants dissolved in different categories of diluents. A brief review of these extraction studies is summarized in a tabular form and shown in Table 1 to have a clear understanding of the various reactive systems used.

2.1. Equilibrium Models. The theories of equilibria of reactive extraction are explained in this section. The model describes the physical and chemical phenomena occurring in the extraction process in the mathematical forms. They also explain interaction mechanism between the components of aqueous (acid and water) and organic (extractant and/or diluent) phases. In these model equations, assumption is made that all the reactions are in thermodynamic equilibrium occurring at the interface of aqueous and organic phases.

2.1.1. Mass Action Law Model. Equilibrium data are interpreted by Mass Action Law, which was proposed by Guldberg and Waage in 1864. Kertes and King in 1986 [3] applied Mass Action Law for reactive extraction of carboxylic acids. In the Mass Action Law model, activities of the aqueous and organic phase species are assumed to be proportional to the respective concentration of the species and the equilibrium constant takes care of the constant of proportionality or the nonidealities associated with the reactive system. Therefore, the apparent equilibrium constant (written in terms of species concentration) is used for the development of mathematical model of the reaction equilibrium. This model can be subcategorized in two types: (i) physical extraction where only

Table 1: Extractant/diluent system for the recovery of carboxylic acid by reactive extraction: equilibrium studies.

SL number	Carboxylic acid	Extractant	Diluent	Parameters studied	Findings	References
1	Different carboxylic acids	Organophosphorous and aliphatic amines	Alcohols, ketones, ethers, and hydrocarbons	Various aqueous and organic phase parameters	Reviewed the extraction chemistry of carboxylic acids	[3]
2	Lactic acid	TOA	Hexane	Type of acid and initial acid concentration	Hydrophobicity of the acid controls equilibrium constants	[20]
3	Acetic, lactic, succinic, malonic, fumaric, and maleic	Tri-alkyl amine (Alamine 336)	Methyl isobutyl ketone (MIBK), n-heptane, dichloromethane (DCM), and nitrobenzene	Effects of diluent-complex interactions	Equilibrium constants, partition coefficient and dimerization constant determined	[13]
4	Citric	Alamine 336	*p*-Xylene, toluene, benzene, MIBK, 1-octanol, DCM, and chloroform	Effect of diluents	K_{11}, K_{12} and K_{23} are correlated with solvatochromic parameters	[12]
6	Lactic	TOA	Xylene	Temperature, acid and TOA concentration	(1 : 1), (1 : 2), and (3 : 1) acid-TOA complexes formed	[21]
7	(L+) lactic	Tri-propyl amine (TPA) and TOA	1-octanol and heptane	Acid and amine concentration	Mixed tertiary amine of short and long chain can facilitate easy phase separation	[22]
8	Citric, lactic, and malic	Tri-iso-octyl amine (TIOA)	1-octanol and heptane	Effect of modifier and type of acid	Extraction mechanism is proposed considering physical and chemical extraction	[23]
9	Glyoxylic, glycolic, acrylic, and benzoic	Tri-alkyl phosphine oxide (TRPO)	Kerosene	Aqueous and organic phase compositions	Equilibrium model for K_{11} is proposed	[24]
10	Propionic	Aliquat 336	Cyclohexane, hexane, toluene, MIBK, ethyl acetate, hexane + MIBK, hexane + toluene, and MIBK + toluene	Effect of diluent and diluent mixture, acid and amine concentration	Order of extraction: ethyl acetate > MIBK > MIBK + toluene > toluene > hexane + MIBK > toluene + hexane > cyclohexane > hexane	[25]
11	Propionic	Tri-*n*-butyl phosphate (TBP), TOA, and Aliquat 336	1-Octanol	Acid and extractant concentration	Order of extractibility is found to be TOA > Aliquat 336 > TBP, 1 : 1 complex formed	[26]

Table 1: Continued.

SL number	Carboxylic acid	Extractant	Diluent	Parameters studied	Findings	References
12	Itaconic, maleic, malic, oxalic, tartaric, and succinic	TBP	Do-decane	pH and initial acid concentration	Mechanism of di-carboxylic acids is proposed	[27]
13	Nicotinic	TOPO and TBP	Benzene, heptane, kerosene, 1-octanol, MIBK, diethyl ether, decane, kerosene + 1-octanol, and heptane + 1-octanol	Effect of diluent, extractant type, initial acid, and extractant concentration	Solvation number and equilibrium extraction constants are determined	[28]
14	Levulinic	*n*-Lauryl tri-alkyl-methyl amine (Amberlite LA-2)	Dimethyl phthalate, dimethyl adipate, dimethyl succinate, dimethyl glutarate, diethyl carbonate, isoamyl alcohol, 1-hexanol, 1-octanol, 1-nonanol, 1-decanol, diisobutyl ketone (DIBK), and MIBK	Diluent effect, and amine concentration	LSER model proposed and K_{11}, K_{21}, and K_{31} are determined	[29]
15	Formic	Amberlite LA-2	Dimethyl phthalate, dimethyl adipate, dimethyl succinate, dimethyl glutarate, diethyl carbonate, isoamyl alcohol, 1-hexanol, 1-octanol, 1-nonanol, 1-decanol, DIBK, and MIBK	Effect of diluent and amine concentration	Extraction constants (K_{51}, K_{61}, and K_{71}) were determined and LSER model proposed	[30]
16	Nicotinic	Organophosphorous and aliphatic amines	Alcohols, ketones, ethers, and hydrocarbons	Various aqueous and organic phase parameters	Reviewed the extraction chemistry of nicotinic acid	[31]
17	Glycolic	Amberlite LA-2	1-Octanol, cyclohexane, iso-octane, toluene, 2-octanone, and MIBK	Solvent type, amine and acid concentration	Order of extraction: MIBK > 2-octanone > 1-octanol > toluene > iso-octane > cyclohexane	[32]
18	Citric	Tri-dodecylamine (TDDA) and Amberlite LA-2	MIBK, 1-octanol, toluene, cyclohexane, 1-octanol + toluene, 1-octanol + MIBK, MIBK + toluene, 1-octanol + toluene, 1-octanol + MIBK, MIBK + toluene, and iso-octane	Diluent effect, amine and acid concentration	1-Octanol proposed to be most effective solvent	[33]
19	Glutaric	TOA	Isoamyl alcohol, 1-octanol, 1-nonanol, 1-decanol, methyl ethyl ketone (MEK), diisobutyl ketone (DIBK), hexan-2-one, toluene, kerosene, and hexane	Diluent effect, amine and acid concentration	Kerosene is found to be the most effective diluent. Equilibrium constants for 1 : 1 and 2 : 1 complex are estimated.	[34]

Table 1: Continued.

SL number	Carboxylic acid	Extractant	Diluent	Parameters studied	Findings	References
20	Lactic	TBP	Dodecane	Acid and solvent composition, change in the phase volumes and pH	Apparent equilibrium constants and the number of reacting extractant molecules	[35]
21	Acrylic	Amberlite LA-2	Cyclohexane, 2-octanone, toluene, MIBK, iso-octane, hexane, and 1-octanol	Type of diluent, and amine concentration	1 : 1 & 1 : 2 acid-amine complexes for proton-donating diluents and 1 : 1 & 2 : 3 for non-proton-donating diluents, overall extraction constants (K_{11}, K_{12} and K_{23})	[36]
22	Formic	TDDA and TBP	Ethyl valerate, diethyl adipate, diethyl sebacate, 1-octanol, and heptane	Effect of diluent, acid and amine concentration	Comparative study of physical and chemical extraction, TDDA suggested to be the best extractant	[37]
23	Penicillin G	Di-*n*-octylamine, TOA, N235 (a mixture of tertiary amines), TBP, and di-(2-ethylhexyl) phosphoric acid (D2EHPA)	n-Butyl acetate, MIBK, 2-ethyl hexanol, kerosene, and heptane	Initial acid concentration, pH and temperature	Effect of extractant on the stability of penicillin G mainly depends on temperature, degradation of penicillin G in alkali solution is governed by pH, mechanism of degradation discussed	[38]
24	Succinic	Amberlite LA-2	Hexane, cyclohexane, toluene, iso-octane, MIBK, 2-octanone, and 1-octanol	Initial extractant concentration	Extraction constants (1 : 1, 1 : 2 & 2 : 3) found out, order of extraction of diluents: 1-octanol > 2-octanone > MIBK > toluene > iso-octane > hexane > cyclohexane	[39]
25	Itaconic	TBP and Aliquat 336	Sunflower oil	Initial acid and extractant concentration	Non toxic system proposed	[40]
26	Propionic	TBP	Kerosene and 1-decanol	Aqueous and organic phase compositions, and temperature	Modifier has a strong effect on degree of extraction	[41]

Table 1: Continued.

SL number	Carboxylic acid	Extractant	Diluent	Parameters studied	Findings	References
27	L (+) Tartaric	Amberlite LA-2	1-octanol, cyclohexane, isooctane, hexane, and MIBK	Organic phase compositions	Extractability of acid is high especially with polar solvents (MIBK and 1-octanol)	[42]
28	Caproic	TBP	MIBK and xylene	Phase compositions	MIBK is a better solvent than xylene	[43]
29	Acetic	TOA	DCM, butyl acetate, heptanes, and 1-octanol	Organic phase compositions and pH	The solvent polarity controls the formed structure of the interfacial acid and TOA compounds	[44]
30	Picolinic	TBP	Sunflower and castor oil	Aqueous and organic phase compositions	Different models are used to represent the equilibrium data	[45]
31	Picolinic	Trialkylamine (N235), TBP	Tetrachloromethane, kerosene, and 1-octanol	Aqueous and organic phase compositions, and pH	Distribution coefficient highly dependent on pH and the apparent alkalinity of N235/1-octanol	[46]
32	Acetic, propionic, butyric and valeric	TBP	Cyclohexane, sulfonated kerosene, and 1-octanol	Equilibrium time, temperature and phase ratio	Conditions of extraction and stripping are found	[47]
33	Citric	TOA	Rice bran oil, sunflower oil, soybean oil, and sesame oil	Aqueous and organic and phase compositions	Overall extraction constants and association numbers	[48]

diluent (pure form) is used for extraction and (ii) chemical extraction where both extractant (phosphoric and aminic) and diluent take part in the extraction process.

(1) Physical Extraction. The process of physical equilibria with pure diluent takes place in three parts: (i) partial dissociation of the acid molecule in aqueous phase, (ii) distribution of the undissociated acid molecule between aqueous and organic phases, and (iii) dimerization of undissociated acid molecule in the organic phase.

(i) Carboxylic acid can exist in undissociated (HC) and dissociated (C^-) forms in the aqueous solution of water. The dissociation of the acid in the aqueous solution depends upon the strength of the acid (pK_a) and is described by

$$HC \longleftrightarrow H^+ + C^- \tag{1}$$

The dissociation constant (K_a) is calculated using

$$K_a = \frac{[H^+][C^-]}{[HC]}. \tag{2}$$

The total acid concentration in the aqueous phase (C_{HC}) and undissociated acid [HC] concentration can be expressed as (3) and (4), respectively:

$$C_{HC} = [HC] + [C^-] \tag{3}$$

$$[HC] = \frac{C_{HC}}{(1 + (K_a/[H^+]))}. \tag{4}$$

(ii) The distribution of the undissociated acid molecule between aqueous and organic phases is represented by (5) and the corresponding partition coefficient is given by (6):

$$HC \longleftrightarrow \overline{HC} \tag{5}$$

$$P = \frac{\overline{[HC]}}{[HC]}. \tag{6}$$

(iii) The undissociated extracted acid molecules can dimerize in the organic phase due to the strong donor-acceptor interaction. This is due to the solute-solute interaction through hydrogen bond which is stronger than the solute-solvent interaction. The dimerization of the undissociated extracted acid in the organic phase ($\overline{HC}$) is represented by

$$2\overline{HC} \longleftrightarrow \left(\overline{HC}\right)_2 \tag{7}$$

The dimerization constant (D) is expressed by

$$D = \frac{\overline{(HC)_2}}{\overline{(HC)}^2}. \tag{8}$$

The extraction efficiency (with pure diluent) of acid is calculated by the physical distribution coefficient, K_D^{diluent}:

$$K_D^{\text{diluent}} = \frac{\overline{C}_{HC}^{\text{diluent}}}{C_{HC}^{\text{diluent}}}, \tag{9}$$

where $\overline{C}_{HC}^{\text{diluent}}$ is the total (undissociated and dimer forms) concentration of acid in the organic phase and C_{HC}^{diluent} is the total (dissociated and undissociated) concentration in aqueous phase at equilibrium with pure diluent.

For a dilute concentration of acid (as used in this study), K_D^{diluent} in terms of dimerization constant (D) and partition coefficient (P) can be represented as [3]

$$K_D^{\text{diluent}} = P + 2DP^2[HC]. \tag{10}$$

To estimate the values of physical extraction parameters (P and D), the plots of K_D^{diluent} versus [HC] can be fitted linearly and value of P from the intercept and D from the slope can be obtained.

(2) Chemical Extraction. The interaction of acid molecule with the extractant molecule in the chemical extraction can occur in two ways: (i) through hydrogen bonding of undissociated acid molecule, (11) and (ii) by ion pair formation, (12) [73]:

$$HC + \overline{S} \longleftrightarrow \overline{(HC)(S)} \tag{11}$$

$$H^+ + C^- + \overline{S} \longleftrightarrow \overline{H^+C^-S} \tag{12}$$

The extraction mechanism described in (11) and (12) depends on the pH of aqueous solution, pK_a of acid, the acid and extractant concentrations, and the basicity of the extractant with respect to the acid (pK_B). The extraction of carboxylic acid by phosphorous based extractants (TBP, TOPO, etc.) can be described by (11) and that for amine based extractants (TOA, Aliquat 336, etc.) by both mechanisms (11) and (12).

An equilibrium extraction process is described as a set of reactions (see (13)) between m molecules of acid (HC) and n molecules of extractant (S) to form various ($m:n$) complexes with corresponding apparent equilibrium constant (K_E) as given by (14):

$$m\text{HC} + n\overline{S} \longleftrightarrow \overline{(HC)_m(S)_n} \tag{13}$$

$$K_E = \frac{\left[\overline{(HC)_m(S)_n}\right]}{[HC]^m\left[\overline{S}\right]^n}. \tag{14}$$

The distribution coefficient (K_D) can be written as

$$K_D = \frac{\overline{C}_{HC}}{C_{HC}} = m\frac{\left[\overline{(HC)_m(S)_n}\right]}{C_{HC}}. \tag{15}$$

Substituting the values of [HC] and $[\overline{(HC)_m(S)_n}]$ from (4) and (15), respectively, in (14) results in

$$K_E = \frac{K_D(1 + K_a/[H^+])^m}{mC_{HC}^{m-1}\left[\overline{S}\right]^n}. \tag{16}$$

The equilibrium free extractant concentration ($[\overline{S}]$) in the organic phase is represented as

$$[S] = \left[\overline{S}\right]_{\text{in}} - n\left[\overline{(HC)_m(S)_n}\right] \tag{17}$$

$$\left[\overline{S}\right] = \left[\overline{S}\right]_{\text{in}} - \frac{K_D n C_{HC}}{m}. \tag{18}$$

Now, putting the value of $[\overline{S}]$ from (18) in (16), (19) is derived:

$$K_D = mK_E\left(\left[\overline{S}\right]_{in} - K_D n\frac{C_{HC}}{m}\right)^n \frac{{C_{HC}}^{m-1}}{\left(1 + K_a/\left[H^+\right]\right)^m}. \quad (19)$$

The values of equilibrium extraction constant (K_E) and the stoichiometry (m, n) of the reactive extraction are determined by minimizing the error between the experimental and predicted values of K_D.

The equilibrium model for the simultaneous formation of various types of acid-extractant complexes (1 : 1, 2 : 1, 3 : 1, 1 : 2, etc.) can be represented by a system of equations (see (20) to (23)) depending upon the type of the acid, extractant, and diluent and their concentrations used in the experiment.

$$HC + \overline{S} \longleftrightarrow \overline{(HC)(S)} \quad (20)$$

$$HC + \overline{(HC)(S)} \longleftrightarrow \overline{(HC)_2(S)} \quad (21)$$

$$HC + \overline{(HC)_2(S)} \longleftrightarrow \overline{(HC)_3(S)} \quad (22)$$

$$\overline{(HC)(S)} + \overline{S} \longleftrightarrow \overline{(HC)(S)_2} \quad (23)$$

The corresponding extraction constants (K_{11}, K_{21}, K_{31} and K_{12}) are calculated using (24) to (27):

$$K_{11} = \frac{\overline{C}_{11}\left(1 + K_a/\left[H^+\right]\right)}{C_{HC}\left[\overline{S}\right]} \quad (24)$$

$$K_{21} = \frac{\overline{C}_{21}\left(1 + K_a/\left[H^+\right]\right)}{C_{HC}\overline{C}_{11}} \quad (25)$$

$$K_{31} = \frac{\overline{C}_{31}\left(1 + K_a/\left[H^+\right]\right)}{C_{HC}\overline{C}_{21}} \quad (26)$$

$$K_{12} = \frac{\overline{C}_{12}}{\overline{C}_{11}\left[\overline{S}\right]}. \quad (27)$$

$\overline{C}_{11}$, $\overline{C}_{21}$, $\overline{C}_{31}$, and $\overline{C}_{12}$ are the concentrations of 1 : 1, 2 : 1, 3 : 1, and 1 : 2 complexes, respectively, in the organic phase. $\overline{C}_{HC}$ and $[\overline{S}]$ are given by (28) and (29), respectively:

$$\begin{aligned}\overline{C}_{HC} &= \overline{C}_{11} + 2\overline{C}_{21} + 3\overline{C}_{31} + \overline{C}_{12} \\ &= \frac{K_{11}\left[\overline{S}\right]C_{HC}}{\left[1 + K_a/\left[H^+\right]\right]} + \frac{2K_{11}K_{21}\left[\overline{S}\right]C_{HC}^2}{\left[1 + K_a/\left[H^+\right]\right]^2} \\ &\quad + \frac{3K_{11}K_{21}K_{31}\left[\overline{S}\right]C_{HC}^3}{\left[1 + K_a/\left[H^+\right]\right]^3} + \frac{K_{11}K_{12}\left[\overline{S}\right]^2 C_{HC}}{\left[1 + K_a/\left[H^+\right]\right]}\end{aligned} \quad (28)$$

$$\begin{aligned}\left[\overline{S}\right] &= \left[\overline{S}\right]_{in} - \left(\overline{C}_{11} + \overline{C}_{21} + \overline{C}_{31} + 2\overline{C}_{12}\right) \\ &= \left[\overline{S}\right]_{in} - \left(\frac{K_{11}\left[\overline{S}\right]C_{HC}}{\left[1 + K_a/\left[H^+\right]\right]} + \frac{K_{11}K_{21}\left[\overline{S}\right]C_{HC}^2}{\left[1 + K_a/\left[H^+\right]\right]^2}\right. \\ &\quad + \frac{K_{11}K_{21}K_{31}\left[\overline{S}\right]C_{HC}^3}{\left[1 + K_a/\left[H^+\right]\right]^3} \\ &\quad \left. + \frac{2K_{11}K_{12}\left[\overline{S}\right]^2 C_{HC}}{\left[1 + K_a/\left[H^+\right]\right]}\right).\end{aligned} \quad (29)$$

Using experimental results and by applying the mass action law, the values of the individual equilibrium constants (K_{11}, K_{21}, K_{31} and K_{12}) and the concentration of complexes ($\overline{C}_{11}$, $\overline{C}_{21}$, $\overline{C}_{31}$, and $\overline{C}_{12}$) can be estimated. An objective function can be defined to determine individual equilibrium constants based on experimental values of $\overline{C}_{HC}$.

Now, a model based on the loading ratio (Z) for formation of various types of complexes (1 : 1, 2 : 1, etc.) between acid and extractant can also be described. The extent to which the organic phase (extractant and diluent) may be loaded with acid is expressed by the loading ratio. It is a ratio of acid concentration in the organic phase at equilibrium to the initial extractant concentration in the organic phase ($[\overline{S}]_{in}$):

$$Z = \frac{\overline{C}_{HC}}{\left[\overline{S}\right]_{in}} \quad (30)$$

The value of Z depends on the extractability of the acid (strength of the acid-base interaction) and its aqueous concentration and the stoichiometry of the overall extraction equilibrium. It is found that when the organic phase is not highly concentrated by acid, that is, at very low loading ratios ($Z < 0.5$), 1 : 1 acid-extractant complex is formed. A plot of $Z/(1 - Z)$ versus [HC] yields a straight line passing through origin with a slope of complexation constant (K_{11}) as given by (31):

$$\frac{Z}{1 - Z} = K_{11}\left[HC\right]. \quad (31)$$

If the carboxylic acid concentration is high enough (at least $Z > 0.5$), this relation can be expressed as shown by

$$\frac{Z}{m - Z} = K_{m1}\left[HC\right]^m. \quad (32)$$

To account for the extraction only by extractant, a term is defined for the distribution of acid by chemical extraction (K_D^{chem}) as

$$K_D^{chem} = \frac{\overline{C}_{HC} - \nu\overline{C}_{HC}^{diluent}}{C_{HC}}, \quad (33)$$

where ν is the volume fraction of diluent in the organic phase.

Therefore, the overall distribution coefficient (K_D^{total}) by physical and chemical extraction is obtained by adding (9) and (33).

$$K_D^{total} = \nu K_D^{diluent} + K_D^{chem} \quad (34)$$

In general the diluent alone also solvates some amount of solute (acid) from aqueous solution by physical extraction which is described in the previous section. Therefore, in such a case, expression for $[\overline{(HC)_m(S)_n}]$ is represented as

$$\left[\overline{(HC)_m\,(S)_n}\right] = \overline{C}_{HC} - \nu\overline{C}_{HC}^{\text{diluent}}. \quad (35)$$

Therefore, (35) could be obtained by including the term for physical extraction and rewriting (34) as

$$K_{mn}\,[HC]^m = \frac{\overline{C}_{HC} - \nu\overline{C}_{HC}^{\text{diluent}}}{\left[\left[\overline{S}\right]_{in} - n\left(\overline{C}_{HC} - \nu\overline{C}_{HC}^{\text{diluent}}\right)\right]^n}. \quad (36)$$

2.1.2. Linear Solvation Energy Relation (LSER). A linear solvation energy relationship (LSER) approach has been introduced by Kamlet et al. [74] in 1983 and then improved by Abraham [75]. It characterizes solvation effects in terms of nonspecific and H-bonding interactions. Thus, a solvation property of interest (XYZ) for an organic solute is modeled by a linear solvation energy relationship of the form [76]

$$XYZ = XYZ^0 + s\left(\pi^* + d\delta\right) + a\alpha + b\beta + h\delta_H + e\xi, \quad (37)$$

where δ_H is the Hildebrand's solubility parameter, a measure of the solvent-solvent interactions that are interrupted in creating a cavity for the solute. π^*, an index of solvent dipolarity or polarizability, measures the ability of the solvent to stabilize a charge or a dipole by virtue of its dielectric effect. δ, a polarizability correction term, equals 0.0 for nonchlorinated aliphatic solvents, 0.5 for polychlorinated aliphatics, and 1.0 for aromatic solvents. Solvatochromic parameter β, representing scale of solvent HBD (hydrogen-bond donor) acidities, describes the ability of solvent to donate a proton in a solvent-to-solute H-bond. α, scale of HBA (hydrogen-bond acceptor) basicities, provides a measure of the solvent's ability to accept a proton (donate an electron pair) in a solute-to-solvent H-bond. The ξ parameter, a measure of coordinate covalency, equals −0.20 for P=O bases, 0.0 for C=O, S=O, and N=O bases, 0.20 for single-bonded oxygen bases, 0.60 for pyridine bases, and 1.00 for sp^3-hybridized amine bases. The coefficients p, s, e, d, a, h, and b are the regression coefficients that measure the relative susceptibilities of XYZ to indicated solvent property scale. Equation (37) is adopted to describe the effect of diluents on the values of distribution coefficients (K_D):

$$\log_{10}K_D = \log_{10}K_D{}^0 + s\left(\pi^* + d\delta\right) + a\alpha + b\beta + h\delta_H + e\xi, \quad (38)$$

where the parameters π^*, δ, β, and α refer to the diluents, and $K_D{}^0$ represents the distribution coefficient for an ideal diluent. The fifth term of (38), which contains the solubility parameter δ_H, does not affect the values of the objective function ($\log_{10}K_D$) significantly and the value of $\xi = 0$ is considered for the diluents used in this study. Thus, (38) results in

$$\log_{10}K_D = \log_{10}K_D{}^0 + s\left(\pi^* + d\delta\right) + b\beta + a\alpha. \quad (39)$$

Equation (39) can be adopted to describe the effect of diluents on the values of distribution coefficients (K_D). In case, a mixture of diluents is used with the extractant, the solvatochromic parameters of the solvent mixtures are calculated as [77]

$$SP_{12} = X_1SP_1 + \left(1 - X_1\right)SP_2, \quad (40)$$

where X_1 is the mole fraction of the first solvent and $X_2 = 1 - X_1$ is the mole fraction of the second solvent. SP_1 is the solvatochromic parameter of the first solvent and SP_2 is the solvatochromic parameter of the second solvent in solvent mixtures. The solvatochromic parameters of different diluents can be found in the study by Kamlet et al. [74].

2.2. Kinetic Studies on Reactive Extraction of Carboxylic Acids. Kinetic studies are equally essential as equilibrium studies for the complete design of a reactive extraction unit. Lewis type stirred cell [78], cylindrical stirring vessel with highly agitated system [50], stirred cell with a microporous hydrophobic membrane [79], and so forth were used to perform the kinetic studies on the reactive extraction of various carboxylic acids. In these studies, the investigators have described and analyzed the kinetic mechanism of reactive extraction using formal elementary kinetic model, a mechanism of reactions of acid-amine complexes [50], theory of extraction accompanied by a chemical reaction [79], and so forth. The estimation of the intrinsic kinetic parameters such as rate constants (forward and backward) and reaction order were also carried out using experimental data. According to their findings, the reaction between the acid and the extractant not only depends on the composition of the organic and aqueous phases, it also depends on the hydrodynamic parameters (volume ratio of phases, interfacial area and speed of agitation) of the system which also confirmed the region of the (mass transfer controlled or reaction controlled) reaction. In a study by Jun et al. [80] in 2007, it was observed that the reaction rates were affected by pH and contamination present in the aqueous phase. At a pH greater than the pK_a of acid, more dissociation took place leading to the reduction in the extraction efficiency. Therefore, it was recommended that, to have an effective separation of acid from the production media, the pH of the fermentation broth should be kept at a value less than the pK_a of the acid. Further, a brief review of the kinetic studies on reactive extraction is summarized in Table 2 to have an overview of the reactive kinetics of different carboxylic acids.

2.2.1. Kinetic Model. A comprehensive study on the theory of extraction accompanied with chemical reaction in a stirred cell is proposed by Doraiswamy and Sharma [79] in 1984 to determine the effect of chemical reaction on the specific rate of reaction. With the help of the film and renewal theories with physico-chemical and hydrodynamic parameters, they have classified the reactive system into four reaction regimes (very slow, slow, fast, and instantaneous) depending on their relative diffusion and reaction rates (Table 3).

The value of physical mass transfer coefficient (k_L) is required to confirm the regime of reaction. This is obtained

Table 2: Extractant/diluent system for the separation of carboxylic acids by reactive extraction: kinetic studies.

SL number	Carboxylic acid	Extractant	Diluent	Parameters	Findings	References
1	Penicillin G	Amberlite LA-2	Kerosene	Agitation speed, interfacial area between two immiscible solutions, pH of aqueous phase, initial carrier, and penicillin G concentration	A rate equation for the mass transfer was proposed with the forward and backward rate constants as $k_1 = 1.64\ L^3\ mol^{-2}\ s^{-1}$ and $k_2 = 6.56 \times 10^{-5}\ L\ s^{-1}$, respectively	[49]
2	Citric	TOA	iso-decanol and n-paraffin	Concentrations of acid and amine and speed of agitation	Formal elementary kinetic model proposed and reaction kinetics evaluated	[50]
3	Penicillin G	Amberlite LA-2	Kerosene and n-butyl acetate	Aqueous and organic phase compositions, pH, and temperature	The fractional resistances of aqueous layer diffusion, interfacial chemical reaction, and organic layer diffusion were quantitatively determined	[51]
4	Tartaric	TIOA	iso-decanol, and kerosene	Concentrations of acid, amine, and iso-decanol	Modified Langmuir isotherm was proposed and kinetic parameters interpreted by a formal elementary kinetic model	[52]
5	Lactic	Aliquat 336	Oleyl alcohol	Initial lactate and extractant concentrations in extraction, initial chloride, and extractant-lactate complex concentrations in stripping	Extraction and stripping kinetics were investigated, diffusion through the organic-phase film was determined as the rate-determining step	[53]
6	Phenyl acetic	Alamine 336	Kerosene and MIBK	Acid and amine concentration, volume ratio of phases, and stirrer speed	Intrinsic kinetics were described; the reaction found to be zero order in Alamine 336 and first order in acid with a rate constant of $0.9\ s^{-1}$	[54]
7	Penicillin G	Amberlite LA-2	Kerosene	Acid and amine concentration, and pH	Dispersed liquid-liquid extraction system proposed; Danckwert and Biot nos used to determine rate step	[55]

Table 3: Classical limiting regimes for irreversible reaction in a stirred cell.

Regime	Description	Hatta Number (Ha)	Effect on the specific rate of extraction ($mol{\cdot}m^{-2}{\cdot}s^{-1}$)			
			$\overline{[HC]}$ $mol{\cdot}L^{-1}$	$[S]$ $mol{\cdot}L^{-1}$	Stirrer speed (N, rpm)	Volume ratio of phases
1	Very slow	<<1	$\alpha[\overline{HC}]^m$	$\alpha[\bar{S}]^n$	None	$\alpha\ V_{org}$
2	Slow		$\alpha[\overline{HC}]$	None	Increases with increase in the speed of stirring	None
3	Fast	>>1	$\alpha[\overline{HC}]^{(m+1)/2}$	$\alpha[\bar{S}]^{n/2}$	None	None
4	Instantaneous		None	$\alpha[S]$	Increases with increase in the speed of stirring	None

by conducting physical extraction of acid from aqueous phase with pure diluent. For a batch process a differential mass balance yields the following equation:

$$V_{aq}\frac{dC_{org}}{dt} = k_L A_c \left(C^*_{org} - C_{org}\right), \tag{41}$$

where A_c is the interfacial area (m^2); V_{aq} is the aqueous phase volume (m^3); C^*_{org} is the equilibrium acid concentration in the organic phase. The time-dependent concentration of acid in the organic phase is obtained by integrating (42) as

$$\ln\left(\frac{C^*_{org}}{C^*_{org} - C_{org}}\right) = \frac{k_L A_c}{V_{org}}t. \tag{42}$$

A plot of $\ln(C^*_{org}/(C^*_{org} - C_{org}))$ versus time (t) yields a straight line and the slope is used to evaluate physical mass transfer coefficient (k_L).

The reaction between acid and extractant is reversible. This problem of reversibility can be avoided by measuring the initial specific rate of reaction which is governed only by the forward reaction. Therefore, in this study, the initial specific rate of extraction, $R_{HC,0}$ ($mol{\cdot}m^{-2}{\cdot}s^{-1}$) is calculated from experimental data using the following equation:

$$R_{HC,0} = \frac{V_{org}}{A_c}\left(\frac{dC_{HC,org}}{dt}\right)_{t=0}. \tag{43}$$

$(dC_{HC,org}/dt)_{t=0}$ is the initial slope of curve which is a representation of the concentration in the organic phase versus time (t). The values of $R_{HC,0}$ are determined with various experimental conditions and used to determine the probable effect of the important variables and to draw an inference on the appropriate kinetics of reactive extraction. In this regard, the effects of the speed of agitation (N) and volume ratio of the phases (V_{org}/V_{aq}) on the initial specific rate of extraction must be examined to determine the reaction regime. Therefore, based on the guidelines provided by Doraiswamy and Sharma [79], the reactive extraction of acid with extractant in diluent is governed by the following equation:

$$R_{HC,0} = k_{\alpha'\beta'}\left[\overline{HC}\right]^{\alpha'}\left[\bar{S}\right]^{\beta'}, \tag{44}$$

where α' and β' are the orders of the reaction with respect to acid and extractant, respectively, and $k_{\alpha'\beta'}$ is the rate constant of the reaction.

For a (α', β') reaction taking place in the organic phase with a rate law shown in (44) and with a high excess of extractant, *Hatta* number (Ha) is given by a general expression as

$$Ha = \frac{\sqrt{\left(2/\left(\alpha'+1\right)\right)k_{\alpha'\beta'}\ \left[\overline{HC}\right]^{\alpha'-1}\left[\bar{S}\right]^{\beta'}\ D_{HC}}}{k_L}. \tag{45}$$

D_{HC} is the diffusion coefficient of acid into diluent. The value of D_{HC} is estimated using Wilke-Change (1955) and Reddy-Doraiswamy (1967) equations which are given by (46) and (47), respectively:

$$D_{HC} = 7.4\times10^{-12}\frac{T\sqrt{\Psi M}}{\eta\left(\forall^{acid}\right)^{0.6}} \tag{46}$$

$$D_{HC} = 10^{-11}\frac{T\sqrt{M}}{\eta\left(\forall^{diluent}\forall^{acid}\right)^{1/3}}, \tag{47}$$

where Ψ denotes the diluent association factor; $\forall$ signifies the molar volume of the component; T is temperature (K); M and η represent molecular weight ($kg{\cdot}kmol^{-1}$) and viscosity ($kg{\cdot}m^{-1}{\cdot}s^{-1}$) of the diluent, respectively.

To determine the effect of reaction on the pure mass transfer of acid from the aqueous to the organic phase, the enhancement factor for the reactive extraction of acid is calculated using

$$\Phi = \frac{R_{HC,0}}{k_L C^*_{org}}. \tag{48}$$

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] H. Bart, *Reactive Extraction*, Springer, Berlin, Germany, 1st edition, 2001.

[2] R. Marr and H. J. Bart, "Solvent-extraction," *Chemie Ingenieur Technik*, vol. 54, no. 2, pp. 119–129, 1982.

[3] A. S. Kertes and C. J. King, "Extraction chemistry of fermentation product carboxylic acids," *Biotechnology and Bioengineering*, vol. 28, no. 2, pp. 269–282, 1986.

[4] C. H. Holten, *Lactic Acid: Properties and Chemistry of Lactic Acid and Derivatives*, Springer, Berlin, Germany, 1st edition, 1971.

[5] R. Wennersten, "Extraction of carboxylic acid from fermentation broth in using solution of tertiary amine," *Journal of Chemical Technology and Biotechnology*, no. 2, pp. 85–94, 1983.

[6] J. Hartl and R. Marr, "Extraction processes for bioproduct separation," *Separation Science and Technology*, vol. 28, no. 1–3, pp. 805–819, 1993.

[7] D. Cascaval and A. I. Galaction, "New separation techniques on bioseparations 1. Reactive extraction," *Chemical Industry*, vol. 58, no. 9, pp. 375–386, 2004.

[8] K. L. Wasewar, A. B. M. Heesink, G. F. Versteeg, and V. G. Pangarkar, "Reactive extraction of lactic acid using alamine 336 in MIBK: equilibria and kinetics," *Journal of Biotechnology*, vol. 97, no. 1, pp. 59–68, 2002.

[9] K. L. Wasewar, A. B. M. Heesink, G. F. Versteeg, and V. G. Pangarkar, "Equilibria and kinetics for reactive extraction of lactic acid using Alamine 336 in decanol," *Journal of Chemical Technology & Biotechnology*, vol. 77, no. 9, pp. 1068–1075, 2002.

[10] S. Kumar and B. V. Babu, "Process intensification for separation of carboxylic acids from fermentation broths using reactive extraction," *Journal of Future Engineering and Technology*, vol. 3, pp. 21–28, 2008.

[11] J. M. Wardell and C. J. King, "Solvent equilibria for extraction of carboxylic acids from water," *Journal of Chemical & Engineering Data*, vol. 23, no. 2, pp. 144–148, 1978.

[12] V. Bízek, J. Horáček, R. Řeřicha, and M. Koušová, "Amine extraction of hydroxycarboxylic acids. 1. Extraction of citric acid with 1-octanol/n-heptane solutions of trialkylamine," *Industrial & Engineering Chemistry Research*, vol. 31, no. 6, pp. 1554–1562, 1992.

[13] J. A. Tamada, A. S. Kertes, and C. J. King, "Extraction of carboxylic acids with amine extractants. 1. Equilibria and law of mass action modeling," *Industrial and Engineering Chemistry Research*, vol. 29, no. 7, pp. 1319–1326, 1990.

[14] D. H. Han and W. H. Hong, "Reactive extraction of lactic acid with trioctylamine/methylene chloride/n-hexane," *Separation Science and Technology*, vol. 31, no. 8, pp. 1123–1135, 1996.

[15] E. Kahya, E. Bayraktar, and Ü. Mehmeto, "Optimization of process parameters for reactive lactic acid extraction," *Turkish Journal of Chemistry*, vol. 25, no. 2, pp. 223–230, 2001.

[16] J. A. Tamada and C. J. King, "Extraction of carboxylic acids with amine extractants. 3. Effect of temperature, water coextraction, and process considerations," *Industrial and Engineering Chemistry Research*, vol. 29, no. 7, pp. 1333–1338, 1990.

[17] V. M. Yabannavar and D. I. C. Wang, "Strategies for reducing solvent toxicity in extractive fermentations," *Biotechnology and Bioengineering*, vol. 37, no. 8, pp. 716–722, 1991.

[18] L. J. Poole and C. J. King, "Regeneration of carboxylic acid-amine extracts by back-extraction with an aqueous solution of a volatile amine," *Industrial & Engineering Chemistry Research*, vol. 30, no. 5, pp. 923–929, 1991.

[19] H. Bart, "From single droplet to column design," in *Proceedings of the 6th World Congress of Chemical Engineering*, Melbourne, Australia, September 2001.

[20] R. S. Juang and R. H. Huang, "Kinetic studies on lactic acid extraction with amine using a microporous membrane-based stirred cell," *Journal of Membrane Science*, vol. 129, no. 2, pp. 185–196, 1997.

[21] R. S. Juang and R. H. Huang, "Equilibrium studies on reactive extraction of lactic acid with an amine extractant," *Chemical Engineering Journal*, vol. 65, no. 1, pp. 47–53, 1997.

[22] Y. K. Hong and W. H. Hong, "Reactive extraction of lactic acid with mixed tertiary amine extractants," *Biotechnology Techniques*, vol. 13, no. 12, pp. 915–918, 1999.

[23] G. Malmary, J. Albet, A. Putranto, H. Hanine, and J. Molinier, "Recovery of aconitic and lactic acids from simulated aqueous effluents of the sugar-cane industry through liquid-liquid extraction," *Journal of Chemical Technology and Biotechnology*, vol. 75, no. 12, pp. 1169–1173, 2000.

[24] Y. Li, Y. Wang, Y. Li, and Y. Dai, "Extraction of glyoxylic acid, glycolic acid, acrylic acid, and benzoic acid with trialkylphosphine oxide," *Journal of Chemical and Engineering Data*, vol. 48, no. 3, pp. 621–624, 2003.

[25] H. Uslu and I. Inci, "(Liquid + liquid) equilibria of the (water + propionic acid + Aliquat 336 + organic solvents) at T = 298.15 K," *Journal of Chemical Thermodynamics*, vol. 39, no. 5, pp. 804–809, 2007.

[26] A. Keshav, K. L. Wasewar, and S. Chand, "Extraction of propionic acid using different extractants (tri-n-butylphosphate, tri-n-octylamine, and Aliquat 336)," *Industrial and Engineering Chemistry Research*, vol. 47, no. 16, pp. 6192–6196, 2008.

[27] G. Kyuchoukov, A. F. Morales, J. Albet, G. Malmary, and J. Molinier, "On the possibility of predicting the extraction of dicarboxylic acids with tributylphosphate dissolved in a diluent," *Journal of Chemical & Engineering Data*, vol. 53, no. 3, pp. 639–647, 2008.

[28] S. Kumar, K. L. Wasewar, and B. V. Babu, "Intensification of nicotinic acid separation using organophosphorous solvating extractants by reactive extraction," *Chemical Engineering and Technology*, vol. 31, no. 11, pp. 1584–1590, 2008.

[29] H. Uslu, Ş. I. Kirbaşlar, and K. L. Wasewar, "Reactive extraction of levulinic acid by amberlite LA-2 extractant," *Journal of Chemical and Engineering Data*, vol. 54, no. 3, pp. 712–718, 2009.

[30] H. Uslu, C. Bayat, S. Gökmen, and Y. Yorulmaz, "Reactive extraction of formic acid by amberlite LA-2 extractant," *Journal of Chemical and Engineering Data*, vol. 54, no. 1, pp. 48–53, 2009.

[31] S. Kumar and B. V. Babu, "Extraction of pyridine-3-carboxylic acid using 1-dioctylphosphoryloctane (TOPO) with different diluents: equilibrium studies," *Journal of Chemical and Engineering Data*, vol. 54, no. 9, pp. 2669–2677, 2009.

[32] Y. S. Asci and I. Inci, "Extraction of glycolic acid from aqueous solutions by amberlite la-2 in different diluent solvents," *Journal of Chemical and Engineering Data*, vol. 54, no. 10, pp. 2791–2794, 2009.

[33] Ş. S. Bayazit, H. Uslu, and I. Inci, "Comparative equilibrium studies for citric acid by amberlite LA-2 or Tridodecylamine (TDA)," *Journal of Chemical & Engineering Data*, vol. 54, no. 7, pp. 1991–1996, 2009.

[34] N. Pehlivanoğlu, H. Uslu, and Ş. I. Kirbaşlar, "Experimental and modeling studies on the extraction of glutaric acid by trioctylamine," *Journal of Chemical and Engineering Data*, vol. 54, no. 12, pp. 3202–3207, 2009.

[35] A. Labbaci, G. Kyuchoukov, J. Albet, and J. Molinier, "Detailed investigation of lactic acid extraction with tributylphosphate dissolved in dodecane," *Journal of Chemical and Engineering Data*, vol. 55, no. 1, pp. 228–233, 2010.

[36] Y. S. Asci and I. Inci, "Extraction equilibria of acrylic acid from aqueous solutions by amberlite LA-2 in various diluents," *Journal of Chemical and Engineering Data*, vol. 55, no. 7, pp. 2385–2389, 2010.

[37] S. Şahin, Ş. S. Bayazit, M. Bilgin, and I. Inci, "Investigation of formic acid separation from aqueous solution by reactive extraction: effects of extractant and diluent," *Journal of Chemical & Engineering Data*, vol. 55, no. 4, pp. 1519–1522, 2010.

[38] Z. Ren, W. Zhang, J. Li, S. Wang, J. Liu, and Y. Lv, "Effect of organic solutions on the stability and extraction equilibrium of penicillin G," *Journal of Chemical and Engineering Data*, vol. 55, no. 8, pp. 2687–2694, 2010.

[39] Y. S. Asci, İ. İnci, and H. Uslu, "LSER modelim extraction of succinic acid by tridodecyl amine dissolved in 2-octanone and 1-octanlol," *Journal of Industrial and Engineering Chemistry*, vol. 32, pp. 1951–1957, 2010.

[40] K. L. Wasewar, D. Shende, and A. Keshav, "Reactive extraction of itaconic acid using tri-n-butyl phosphate and aliquat 336 in sunflower oil as a non-toxic diluent," *Journal of Chemical Technology and Biotechnology*, vol. 86, no. 2, pp. 319–323, 2011.

[41] S. Kumar, D. Datta, and B. V. Babu, "Differential evolution approach for reactive extraction of propionic acid using tri-*n*-butyl phosphate (TBP) in kerosene and 1-decanol," *Materials and Manufacturing Processes*, vol. 26, no. 9, pp. 1222–1228, 2011.

[42] I. Inci, Y. S. Asci, and A. F. Tuyun, "Reactive extraction of L (+) tartaric acid by amberlite LA-2 in different solvents," *E-Journal of Chemistry*, vol. 8, supplement 1, pp. S509–S515, 2011.

[43] K. L. Wasewar and D. Shende, "Equilibrium study for reactive extraction of caproic acid in MIBK and Xylene," *Engineering*, vol. 3, pp. 829–835, 2011.

[44] D. Caşcaval, L. Kloetzer, and A.-I. Galaction, "Influence of organic phase polarity on interfacial mechanism and efficiency of reactive extraction of acetic acid with tri-*n*-octylamine," *Journal of Chemical & Engineering Data*, vol. 56, no. 5, pp. 2521–2526, 2011.

[45] M. D. Waghmare, K. L. Wasewar, S. S. Sonawane, and D. Z. Shende, "Natural nontoxic solvents for recovery of picolinic acid by reactive extraction," *Industrial & Engineering Chemistry Research*, vol. 50, no. 23, pp. 13526–13537, 2011.

[46] L. Zhang, F. Yu, Z. Chang, Y. Guo, and D. Li, "Extraction equilibria of picolinic acid with rrialkylamine/n-octanol," *Journal of Chemical and Engineering Data*, vol. 57, no. 2, pp. 577–581, 2012.

[47] Y.-P. Ren, J.-J. Wang, X.-F. Li, and X.-H. Wang, "Reactive extraction of short-chain fatty acids from synthetic acidic fermentation broth of organic solid wastes and their stripping," *Journal of Chemical & Engineering Data*, vol. 57, no. 1, pp. 46–51, 2012.

[48] A. Keshav, P. Norge, and K. L. Wasewar, "Reactive extraction of citric acid using tri-n-octylamine in nontoxic natural diluents: part 1—equilibrium studies from aqueous solutions," *Applied Biochemistry and Biotechnology*, vol. 167, no. 2, pp. 197–213, 2012.

[49] S. S. Wang and C. J. Lee, "Kinetics of penicillin G extraction by Amberlite LA-2 as a mobile carrier in a constant-interface-area cell," *Chemical Engineering Journal and the Biochemical Engineering Journal*, vol. 58, no. 3, pp. 285–290, 1995.

[50] F. A. Poposka, K. Nikolovski, and R. Tomovska, "Kinetics, mechanism and mathematical modelling of extraction of citric acid with isodecanol/*n*-paraffins solutions of trioctylamine," *Chemical Engineering Science*, vol. 53, no. 18, pp. 3227–3237, 1998.

[51] R.-S. Juang and Y.-S. Lin, "Investigation on interfacial reaction kinetics of Penicillin G and Amberlite LA-2 from membrane flux measurements," *Journal of Membrane Science*, vol. 141, no. 1, pp. 19–30, 1998.

[52] F. A. Poposka, J. Prochazka, R. Tomovska, and A. Grizo, "Extraction of tartaric acid from aqueous solutions with tri-iso-octylamine (HOSTAREX A 324). Equilibrium and kinetics," *Chemical Engineering Science*, vol. 55, no. 9, pp. 1591–1604, 2000.

[53] M. Hironaka, M. Hirata, H. Takanashi, T. Hano, and S. Miura, "Kinetics of lactic acid extraction with quaternary ammonium salt," *Separation Science and Technology*, vol. 36, no. 13, pp. 2927–2943, 2001.

[54] H. K. Gaidhani, K. L. Wasewar, and V. G. Pangarkar, "Intensification of enzymatic hydrolysis of penicillin G: Part 1. Equilibria and kinetics of extraction of phenyl acetic acid by Alamine 336," *Chemical Engineering Science*, vol. 57, no. 11, pp. 1979–1984, 2002.

[55] S. C. Lee, "Kinetics of reactive extraction of Penicillin G by Amberlite LA-2 in kerosene," *AIChE Journal*, vol. 50, no. 1, pp. 119–126, 2004.

[56] J. A. Tamada and C. J. King, "Extraction of carboxylic acids with amine extractants. 2. Chemical interactions and interpretation of data," *Industrial and Engineering Chemistry Research*, vol. 29, no. 7, pp. 1327–1333, 1990.

[57] Y. Tong, M. Hirata, H. Takanashi, T. Hano, M. Matsumoto, and S. Miura, "Solvent screening for production of lactic acid by extractive fermentation," *Separation Science and Technology*, vol. 33, no. 10, pp. 1439–1453, 1998.

[58] A. Keshav, S. Chand, and K. L. Wasewar, "Equilibrium studies for extraction of propionic acid using tri-*n*-butyl phosphate in different solvents," *Journal of Chemical & Engineering Data*, vol. 53, no. 7, pp. 1424–1430, 2008.

[59] S. Kumar and B. V. Babu, "Process intensification of nicotinic acid production via enzymatic conversion using reactive extraction," *Chemical and Biochemical Engineering Quarterly*, vol. 23, no. 3, pp. 367–376, 2009.

[60] Z. Gu, B. Glatz, and C. E. Glatz, "Propionic acid production by extractive fermentation. I. Solvent considerations," *Biotechnology and Bioengineering*, vol. 57, pp. 454–461, 1998.

[61] C. Q. Ma, J. C. Li, J. H. Qiu, M. Wang, and P. Xu, "Recovery of pyruvic acid from biotransformation solutions," *Applied Microbiology and Biotechnology*, vol. 70, no. 3, pp. 308–314, 2006.

[62] T. Kirsch and G. Maurer, "Distribution of binary mixtures of citric, acetic and oxalic acid between water and organic solutions of tri-n-octylamine Part I. Organic solvent toluene," *Fluid Phase Equilibria*, vol. 131, no. 1-2, pp. 213–231, 1997.

[63] R. Canari and A. M. Eyal, "Selectivity in monocarboxylic acids extraction from their mixture solutions using an amine-based extractant: effect of pH," *Industrial and Engineering Chemistry Research*, vol. 42, no. 7, pp. 1301–1307, 2003.

[64] M. Siebold, P. van Frieling, R. Joppien, D. Rindfleisch, K. Schugerl, and H. Roper, "Comparison of the production of lactic acid by three different lactobacilli and its recovery by extraction and electrodialysis," *Process Biochemistry*, vol. 30, no. 1, pp. 81–95, 1995.

[65] T. A. Barbari and C. J. King, "Equilibrium distribution coefficients for extraction of chlorinated hydrocarbons and aromatics from water into undecane," *Environmental Science and Technology*, vol. 16, no. 9, pp. 624–627, 1982.

[66] C. L. Munson and C. J. King, "Factors influencing solvent selection for extraction of ethanol from aqueous solutions," *Industrial & Engineering Chemistry Process Design and Development*, vol. 23, no. 1, pp. 109–115, 1984.

[67] P. D. Mackenzie and C. J. King, "Combined solvent extraction and stripping for removal and isolation of ammonia from sour waters," *Industrial and Engineering Chemistry Process Design and Development*, vol. 24, no. 4, pp. 1192–1200, 1985.

[68] S. T. Yang, S. A. White, and S. T. Hsu, "Extraction of carboxylic acids with tertiary and quaternary amines: effect of pH," *Industrial & Engineering Chemistry Research*, vol. 30, no. 6, pp. 1335–1342, 1991.

[69] J. Procházka, A. Heyberger, V. Bízek, M. Koušová, and E. Volaufová, "Amine extraction of hydroxycarboxylic acids. 2. Comparison of equilibria for lactic, malic, and citric acids," *Industrial and Engineering Chemistry Research*, vol. 33, no. 6, pp. 1565–1573, 1994.

[70] R.-S. Juang, R.-H. Huang, and R.-T. Wu, "Separation of citric and lactic acids in aqueous solutions by solvent extraction and liquid membrane processes," *Journal of Membrane Science*, vol. 136, no. 1-2, pp. 89–99, 1997.

[71] F. A. Poposka, K. Nikolovski, and R. Tomovska, "Equilibria and mathematical models of extraction of citric acid with isodecanol/n-paraffins solutions of trioctylamine," *Journal of Chemical Engineering of Japan*, vol. 30, no. 5, pp. 777–785, 1997.

[72] M. Matsumoto, T. Otono, and K. Kondo, "Synergistic extraction of organic acids with tri-*n*-octylamine and tri-n-butylphosphate," *Separation and Purification Technology*, vol. 24, no. 1-2, pp. 337–342, 2001.

[73] D. Yankov, J. Molinier, J. Albet, G. Malmary, and G. Kyuchoukov, "Lactic acid extraction from aqueous solutions with tri-n-octylamine dissolved in decanol and dodecane," *Biochemical Engineering Journal*, vol. 21, no. 1, pp. 63–71, 2004.

[74] M. J. Kamlet, J. L. M. Abboud, M. H. Abraham, and R. W. Taft, "Linear solvation energy relationships. 23. A comprehensive collection of the solvatochromic parameters, π^*, β and α, and some methods for simplifying the generalized solvatochromic equation," *Journal of Organic Chemistry*, vol. 48, no. 17, pp. 2877–2887, 1983.

[75] M. H. Abraham, "Scales of solute hydrogen-bonding: their construction and application to physicochemical and biochemical processes," *Chemical Society Reviews*, vol. 22, no. 2, pp. 73–83, 1993.

[76] M. H. Abraham, J. M. R. Gola, J. E. Cometto-Muniz, and W. S. Cain, "The solvation properties of nitric oxide," *Journal of the Chemical Society, Perkin Transactions 2*, no. 10, pp. 2067–2070, 2000.

[77] V. Bízek, J. Horáček, and M. Koušová, "Amine extraction of citric acid: effect of diluent," *Chemical Engineering Science*, vol. 48, no. 8, pp. 1447–1457, 1993.

[78] Y.-S. Jun, Y. S. Huh, H. H. Won, and Y. K. Hong, "Kinetics of the extraction of succinic acid with tri-*n*-octylamine in 1-octanol solution," *Biotechnology Progress*, vol. 21, no. 6, pp. 1673–1679, 2005.

[79] L. K. Doraiswamy and M. M. Sharma, *Heterogeneous Reactions: Analysis, Examples, and Reactor Design*, John Wiley & Sons, New York, NY, USA, 1st edition, 1984.

[80] Y.-S. Jun, E. Z. Lee, Y. S. Huh, Y. K. Hong, W. H. Hong, and S. Y. Lee, "Kinetic study for the extraction of succinic acid with TOA in fermentation broth; effects of pH, salt and contaminated acid," *Biochemical Engineering Journal*, vol. 36, no. 1, pp. 8–13, 2007.

Hindawi Publishing Corporation
Journal of Chemistry
Volume 2015, Article ID 198176, 5 pages
http://dx.doi.org/10.1155/2015/198176

Research Article

Experimental and Modeling Studies on the Prediction of Gas Hydrate Formation

Jian-Yi Liu,[1] Jing Zhang,[2,3] Yan-Li Liu,[4] Xiao-Hua Tan,[1] and Jie Zhang[5]

[1] *State Key Laboratory of Oil and Gas Reservoir Geology and Exploitation, Southwest Petroleum University, Xindu Road 8, Chengdu 610500, China*
[2] *Sulige Gas Field Research Center, Changqing Oilfield Company, Xi'an 710018, China*
[3] *National Engineering Laboratory for Exploration and Development of Low Permeability Oil and Gas Fields, Xi'an 710018, China*
[4] *Drilling and Producing Technology Research Institute, Liaohe Oilfield, Panjin 124010, China*
[5] *Tahe Oil Production Plant No.1, Sinopec Northwest Oilfield Branch, Luntai 841600, China*

Correspondence should be addressed to Xiao-Hua Tan; xiaohua-tan@163.com

Received 30 July 2014; Accepted 21 October 2014

Academic Editor: Hasan Uslu

Copyright © 2015 Jian-Yi Liu et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

On the base of some kinetics model analysis and kinetic observation of hydrate formation process, a new prediction model of gas hydrate formation is proposed. The analysis of the present model shows that the formation of gas hydrate not only relevant with gas composition and free water content but also relevant with temperature and pressure. Through contrast experiment, the predicted result of the new prediction method of gas hydrate crystallization kinetics is close to measured result, it means that the prediction method can reflect the hydrate crystallization accurately.

1. Introduction

During the exploitation process, the gas well sometimes closed because of plugging in wellbore or surface pipeline which is caused by gas hydrate [1–4]. In order to solve a series of plugging problems caused by hydrate in oil field and develop natural gas hydrate resources more effectively and utilize hydrate technology more reasonably, the research on natural gas hydrate crystallization kinetics mechanism becomes very necessary [5, 6].

An operation step of crystal nucleus growth process is an important factor need to research in the study of crystallization kinetics [7]. In different system, many factors can be used to control crystal nucleus growth process, such as diffusion, heat transfer, stirring rate, reaction kinetics, and heat exchange rate on surface of crystal [8–10]. In view of the above factors, many researchers propose the crystallization kinetics model which describes the nucleation based on mass transfer theory, crystallization theory, and two-film gas-liquid mass transfer theory [11, 12].

The generation of hydrate is actually the nucleation and crystal growth process. Kinetics of hydrate formation is related to generation rate, pressure, temperature, and so forth. The process can be clearly divided into three steps: at first the generation of crystal nucleus with critical radius, then solid crystal nucleus growth, and at last the components transfer to solid-liquid interface of nuclear at aggregation state [13–15].

Many factors affect the generation of natural gas hydrate; there are three main points. (1) When the gas temperature is equal to or lower than the water dew point and is accompanied with free water or liquid water, then hydrate formed. (2) In condition of certain pressure and gas composition, the temperature is lower than the hydrate formation temperature; then hydrate formed. (3) The high operating pressure will rise the hydrate formation temperature [16].

This paper developed a new prediction method of gas hydrate crystallization kinetics according to the shrinkage bubble model and considering the variation of pressure with time at the same time, to establish a new method for the prediction of natural gas hydrate.

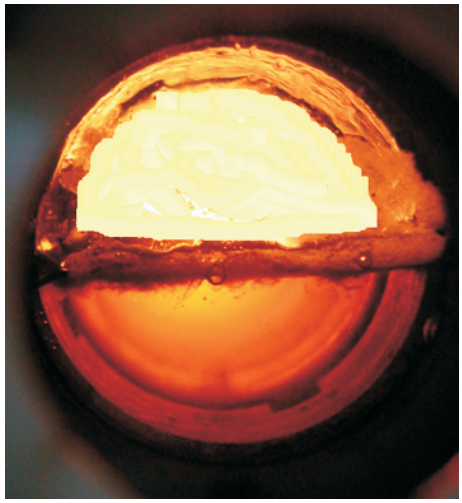


Figure 1: Kinetic observation of hydrate formation process.

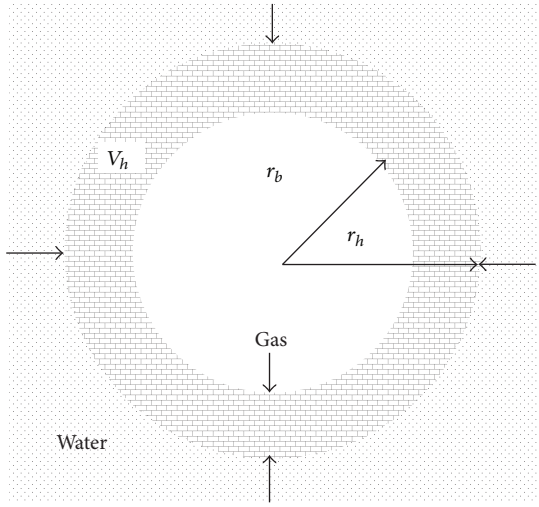


Figure 2: Process of a bubble transforming into hydrate.

2. Model

2.1. Hydrate Growth Dynamic. During the hydrate formation process there is transfer phenomena in the hydrate-gas-water [17–19]. The kinetic observation of hydrate formation process is shown in Figure 1. From the figure we can know that at first hydrate mainly gathered at the air-water interface layer; when the temperature is lower than the hydrate phase equilibrium temperature, hydrate nucleation formed quickly by gas and water molecules inside the bubble [20, 21]. The generation process of crystal nucleus is the process that water diffuse into a bubble and gas in bubble diffuse out of bubble; finally the bubble in hydrate gradually reduced and even disappeared then hydrate crystals formed. Experiments show that the hydrate formation process is controlled by the transmission rate of gas and water molecules, through the establishment of hydrate formation kinetics method can describe the generation process [22].

2.2. Shrinkage Bubble Kinetics. Through the hydrate growth kinetic observation we can know that during the growth process of hydrate crystals the bubbles transform into hydrate; the process is shown in Figure 2. In Figure 2, the exterior is water, the internal is gas, and the ring is hydrate.

Hydrate volume in hydrate crystals is

$$V_h = \frac{4}{3}\pi\left(r_h^3 - r_b^3\right), \tag{1}$$

where r_h is outer radius of hydrate, r_b is bubble radius, and V_h is hydrate volume in hydrate crystals.

The growth rate of hydrate is

$$\frac{dV_h}{dt} = 4\pi\left[r_h^2\frac{dr_h}{dt} - r_b^2\frac{dr_b}{dt}\right]. \tag{2}$$

During the formation process of hydrates, the chemical potential in phase equilibrium systemization is equal. According to gas adsorption model and the law of diffusion, the hydrate formation rate equation is obtained as follows

$$\frac{dV_h}{dt} = 4\pi D\frac{r_b r_h}{r_h - r_b}\left[\frac{p_b - p_d}{p_b}\right], \tag{3}$$

where p_b is inner air pressure of bubble and p_d is decomposition pressure. D is the mass transfer coefficient and it can be expressed as

$$D = \frac{(1 - y_d)\,D_{ha}}{y_d} + \nu c_d D_{hw}, \tag{4}$$

where y_d is lattice occupancy, ν is vacant hole numbers of each water molecule, c_d is water solution concentration under the decomposition pressure of hydrate, D_{ha} is mass transfer coefficient between hydrate and gas, and D_{hw} is mass transfer coefficient between hydrate and water.

Supposing the gas in bubble and hydrate is ideal gas, then

$$V_b p_b + V_h p_{ha}\phi = V_{b0} p_{b0}, \tag{5}$$

where ϕ is porosity and V_b is bubble volume. p_{ha} is the pressure when the gas density in bubble is equal to the gas density in hydrate, ρ_{ha}, which can be expressed as

$$p_{ha} = \frac{\rho_{ha} R_g T}{M}. \tag{6}$$

Because the reaction is controlled by mass transfer, so the change rate of hydrate volume is

$$\omega_h = -\frac{\left[dV_b/dt + (1 - d_{ha})\,(dV_b/dt)\right]}{(V_b + V_h)}. \tag{7}$$

From the experiment we can know that hydrate is plastic materials, so

$$p_1 - p_b = \frac{4}{3} K_c (\omega - c\omega), \tag{8}$$

where ω is compressibility of vacant hole.

Then forward formula becomes

$$\begin{aligned} p_1 - p_b &= \frac{4}{3} K_c \omega_h \left[\frac{r_h^3 - r_b^3}{r_h^3} \right], \\ \frac{dV_b}{dt} &= -\left[\frac{V_b}{p_b} \frac{dp_b}{dt} + \frac{p_{ha}\phi}{p_b} \frac{dV_h}{dt} \right]. \end{aligned} \tag{9}$$

By comprehensive analysis

$$r_h = \left[\frac{3 (V_{b0} p_{b0} - V_b p_b)}{4\pi p_{ha} \phi} + r_b^3 \right]^{1/3}. \tag{10}$$

Dividing the formation process of gas hydrate into many small blocks of time and assuming that the pressure in bubbles is unchanged mean that p_b is a constant. From the above formulas the relationship that r_b changes with time can be known; then the rate of hydrate formation can be known, finally:

$$\begin{aligned} p_1 - p_b = {} & \frac{4}{3} K_c \frac{r_h^3 - r_b^3}{r_h^3} \\ & \times \left(p_b \left[\frac{(p_{ha}\phi / p_b - 1 + d_{ha}) 4\pi D r_b r_h}{(r_h - r_b)(p_b - p_d)/p_h} \right] \right) \\ & \times (V_h p_b + V_{b0} p_{b0} - V_h p_{ha} \phi)^{-1}. \end{aligned} \tag{11}$$

From the above formulas r_h and r_b can be calculated. r_h and r_b are corresponding to p_b, and p_b is corresponding to t, so the relationship between $t - p_b$, r_h, and r_b at the whole reaction time can be calculated.

Through shrinkage bubble kinetics model the relationship between inner air pressure of bubble, outer radius of hydrate, bubble radius, and time can be known. But in the actual conditions, only environmental pressure can be detected, so it is necessary to consider relationship between environmental pressure and time to improve the shrinkage bubble kinetics.

2.3. Improvement of Shrinkage Bubble Kinetics Model. According to the constant volume substance equilibrium rule the alternation law of pressure with time can be known. In the experiments, the volume is constant which is equal to the volume of natural gas and water before hydrate formation and equal to the volume of residual gas, water, and hydrate after hydrate formation; then the pressure change. The process is shown in Figure 3.

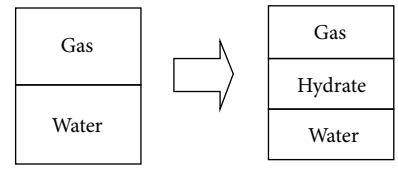


Figure 3: Chart of hydrate formation.

According to the principle of material balance, before hydrate formation there are

$$\begin{aligned} V_{\text{sum}} &= V_g + V_w, \\ V_g &= \frac{ZTp_{\text{sc}} V_{g\text{sc}}}{p_1 T_{\text{sc}}}, \\ V_w &= B_w V_{w\text{sc}}, \end{aligned} \tag{12}$$

where V_g is gas volume in the vessel, m^3. V_w is water volume in the vessel, m^3. Z is Z-factor at the vessel pressure, dimensionless. T is temperature in vessel, K. p_1 is the pressure in vessel before hydrate formation, MPa. T_{sc} is the temperature at standard state, K. p_{sc} is the pressure at standard state, MPa. $V_{g\text{sc}}$ is the injected gas volume at standard state, m^3. $V_{w\text{sc}}$ is the injected water volume at standard state, m^3. B_w is the volume coefficient of water, dimensionless.

After hydrate formation there are

$$\begin{aligned} V_{\text{sum}} &= V_g' + V_w' + V_h, \\ V_w' &= V_w - V_{wc}, \\ p_2 &= \frac{N_h RZT}{V_{\text{sum}} - V_w' - V_h}, \\ V_{wc} &= \frac{KN_h}{M_w \rho_w}, \\ N_h &= \frac{V_h \rho_h}{M_h}, \\ V_h &= n v_h, \\ n &\le \frac{S_{GW}}{\pi (3v_h / 4\pi)^{2/3}}, \end{aligned} \tag{13}$$

where V_g' is gas volume in the vessel, m^3. V_w' is water volume in the vessel, m^3. V_h is hydrate volume in the vessel, m^3. V_{wc} is consumption water volume in the vessel, m^3. p_2 is the pressure in the vessel after hydrate formation, MPa. N_h is molar of generated hydrate, K is molar of water when generate 1 mole hydrate. M_w is hydrate molecular weight, g/mol. ρ is hydrate density, kg/m^3. n is number of generated bubbles. v_h is hydrate volume of each generated bubbles, m^3. S_{GW} is interface area between gas and water, m^2.

Table 1: Gas components.

Components	N_2	CO_2	C_1	C_2	C_3	iC_4	nC_4	iC_5	nC_5	C_6
Content (mol%)	2.012	0.2659	82.9622	9.6578	3.5692	0.3044	0.6639	0.1687	0.2064	0.1894

Table 2: Water analysis.

Item	K^+	Na^+	Ca^{2+}	Mg^{2+}	Cl^-	SO_4^{2-}	HCO_3^-	Total salinity
Content (mg/L)	365	4602	412	96	8007	535	460	14477

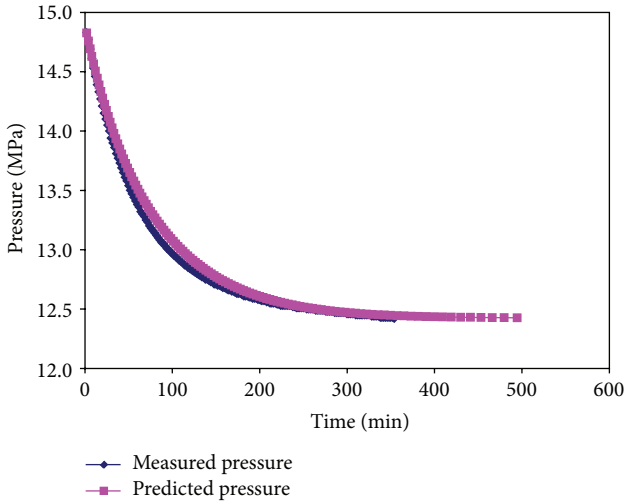


Figure 4: Comparison of the results of prediction method with experiment.

3. Experiment

3.1. Gas Sample. In this experiment using the gas sample, the content of main gas components is measured by gas chromatography, and the components are shown in Table 1.

3.2. Water Sample. In this experiment using the field water, the salinity of formation water is 14477 mg/L and pH value is 5.4. The analysis data of water is shown in Table 2.

Injecting 10 mL formation water into vessel, then injecting gas until the pressure of vessel up to 14.7 MPa, and keeping the temperature at 20°C. It has been known that the temperature of hydrate formation is 18°C when the pressure is 14.7 MPa. By decreasing the temperature to 9°C we can obtain the supercooled degree, that is, 9°C. We observe and record the change of the pressure in the vessel.

4. The Results and Discussions

As shown in Figure 4, at first the pressure in the vessel declined rapidly; then the decrease rate become slow and steady gradually. The predicted pressure is close to measured pressure. But in the actual conditions, only environmental pressure can be detected, so it is just considered relationship between environmental pressure and time to improve the shrinkage bubble kinetics.

5. Conclusion

The predicted result of the new prediction method of gas hydrate crystallization kinetics is close to measured result; it means that the prediction method can reflect the hydrate crystallization accurately. In actual application the various parameters should be modified according to the practical situation.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was jointly supported by the National Natural Science Foundation of China (51474181) and the 2014 Australia China Natural Gas Technology Partnership Fund Top Up Scholarships.

References

[1] E. D. Sloan, *Hydrate Engineering*, Society of Petroleum Engineers, Richardson, Tex, USA, 2000.

[2] I. U. R. F. Makogon, *Hydrates of Hydrocarbons*, Pennwell Books, 1997.

[3] X.-H. Tan, X.-P. Li, and J.-Y. Liu, "Model of continuous liquid removal from gas wells by droplet diameter estimation," *Journal of Natural Gas Science and Engineering*, vol. 15, pp. 8–13, 2013.

[4] X.-H. Tan, J.-Y. Liu, X.-P. Li, G.-D. Zhang, and C. Tang, "A fractal model for the maximum droplet diameter in gas-liquid mist flow," *Mathematical Problems in Engineering*, vol. 2013, Article ID 532638, 6 pages, 2013.

[5] J. Cai, E. Perfect, C.-L. Cheng, and X. Hu, "Generalized modeling of spontaneous imbibition based on hagen-poiseuille flow in tortuous capillaries with variably shaped apertures," *Langmuir*, vol. 30, no. 18, pp. 5142–5151, 2014.

[6] X.-H. Tan, X.-P. Li, J.-Y. Liu, G.-D. Zhang, and L.-H. Zhang, "Analysis of permeability for transient two-phase flow in fractal porous media," *Journal of Applied Physics*, vol. 115, no. 11, Article ID 113502, 2014.

[7] E. D. Sloan Jr. and C. Koh, *Clathrate Hydrates of Natural Gases*, CRC Press, New York, NY, USA, 2007.

[8] W. R. Parrish and J. M. Prausnitz, "Dissociation pressures of gas hydrates formed by gas mixtures," *Industrial & Engineering Chemistry Process Design and Development*, vol. 11, no. 1, pp. 26–35, 1972.

[9] H. Tavasoli, F. Feyzi, M. R. Dehghani, and F. Alavi, "Prediction of gas hydrate formation condition in the presence of thermodynamic inhibitors with the Elliott-Suresh-Donohue Equation of State," *Journal of Petroleum Science and Engineering*, vol. 77, no. 1, pp. 93–103, 2011.

[10] H. Jiang and H. Adidharma, "Prediction of hydrate dissociation conditions for alkanes in the presence of alcohol and electrolyte solutions using ion-based statistical associating fluid theory," *Chemical Engineering Science*, vol. 82, pp. 14–21, 2012.

[11] X.-S. Li, H.-J. Wu, and P. Englezos, "Prediction of gas hydrate formation conditions in the presence of methanol, glycerol, ethylene glycol, and triethylene glycol with the statistical associating fluid theory equation of state," *Industrial & Engineering Chemistry Research*, vol. 45, no. 6, pp. 2131–2137, 2006.

[12] S. Moradi, A. Haghtalab, and A. Fazlali, "Prediction of hydrate formation conditions in the solutions containing electrolyte and alcohol inhibitors and their mixtures using UNIQUAC-NRF models," *Fluid Phase Equilibria*, vol. 349, pp. 61–66, 2013.

[13] H.-J. Ng and D. B. Robinson, "The measurement and prediction of hydrate formation in liquid hydrocarbon-water systems," *Industrial and Engineering Chemistry Fundamentals*, vol. 15, no. 4, pp. 293–298, 1976.

[14] N. Gnanendran and R. Amin, "Modelling hydrate formation kinetics of a hydrate promoter-water-natural gas system in a semi-batch spray reactor," *Chemical Engineering Science*, vol. 59, no. 18, pp. 3849–3863, 2004.

[15] Y. Du and T.-M. Guo, "Prediction of hydrate formation for systems containing methanol," *Chemical Engineering Science*, vol. 45, no. 4, pp. 893–900, 1990.

[16] T.-M. Guo, B.-H. Wu, Y.-H. Zhu, S.-S. Fan, and G.-J. Chen, "A review on the gas hydrate research in China," *Journal of Petroleum Science and Engineering*, vol. 41, no. 1–3, pp. 11–20, 2004.

[17] C. A. Koh, E. D. Sloan, A. K. Sum, and D. T. Wu, "Fundamentals and applications of gas hydrates," *Annual Review of Chemical and Biomolecular Engineering*, vol. 2, pp. 237–257, 2011.

[18] J. Javanmardi, S. Babaee, A. Eslamimanesh, and A. H. Mohammadi, "Experimental measurements and predictions of gas hydrate dissociation conditions in the presence of methanol and ethane-1,2-diol aqueous solutions," *Journal of Chemical and Engineering Data*, vol. 57, no. 5, pp. 1474–1479, 2012.

[19] Y. F. Makogon and R. Y. Omelchenko, "Commercial gas production from Messoyakha deposit in hydrate conditions," *Journal of Natural Gas Science and Engineering*, vol. 11, pp. 1–6, 2013.

[20] M. Mottahedin, F. Varaminian, and K. Mafakheri, "Modeling of methane and ethane hydrate formation kinetics based on non-equilibrium thermodynamics," *Journal of Non-Equilibrium Thermodynamics*, vol. 36, no. 1, pp. 3–22, 2011.

[21] K. Nasrifar and M. Moshfeghian, "A model for prediction of gas hydrate formation conditions in aqueous solutions containing electrolytes and/or alcohol," *The Journal of Chemical Thermodynamics*, vol. 33, no. 9, pp. 999–1014, 2001.

[22] A. Eslamimanesh, A. H. Mohammadi, and D. Richon, "Thermodynamic consistency test for experimental solubility data in carbon dioxide/methane + water system inside and outside gas hydrate formation region," *Journal of Chemical & Engineering Data*, vol. 56, no. 4, pp. 1573–1586, 2011.

Hindawi Publishing Corporation
Journal of Chemistry
Volume 2015, Article ID 836076, 9 pages
http://dx.doi.org/10.1155/2015/836076

Research Article

Layer-by-Layer Assembly of Polysaccharides and 6,10-Ionene for Separation of Nitrogen-Containing Pharmaceuticals and Their Enantiorecognition by Capillary Electrophoresis

Anna Ioutsi, Elena Shapovalova, Aleksandra Prokhorova, and Oleg Shpigun

Analytical Chemistry Division, Department of Chemistry, Lomonosov Moscow State University, Leninskie Gory 1/3, Moscow 119991, Russia

Correspondence should be addressed to Anna Ioutsi; freund-for-me@mail.ru

Received 11 July 2014; Revised 14 October 2014; Accepted 14 October 2014

Academic Editor: Hasan Uslu

Copyright © 2015 Anna Ioutsi et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Two silica capillaries modified layer-by-layer with 6,10-ionene and N-(3-sulfo-3-carboxy)-propionylchitosan (SCPC) and with 6,10-ionene and dextran sulfate (DS) were prepared and investigated. Dynamic coating of the capillary efficiently reduces the adsorption of the background electrolyte, sample matrix components, and analytes on its inner wall. Such coatings effect good reproducibility and sensitivity of determination. We demonstrate that separation of betablockers, calcium channel blockers, alpha-adrenergic agonists, H_1-blockers, and diuretics was the most efficient and rapid separation with a capillary modified with dextran sulfate. Tetrahydrozoline, carbinoxamine, and furacilin, which are commonly employed as treatments for allergic rhinitis, were identified in human urea. Their concentrations, independently verified by HPLC, were found to be 5.3 ± 0.8, 6.6 ± 0.5, and 0.9 ± 0.2 μg mL^{-1}, with LOD = 0.07, 0.03, 0.10 μg mL^{-1}, and LOQ = 1.0, 0.8, 0.6 μg mL^{-1}, respectively.

1. Introduction

Capillary electrophoresis (CE) is increasingly used for the determination of the qualitative and quantitative composition of pharmaceuticals. Medicines are often determined in biological fluids (blood plasma, urea) as part of investigation of their pharmacokinetics and mechanism of action in humans. Successful application of CE to the separation and identification of various compounds and their enantiomers owes to its high efficiency, the possibility to quickly and easily adjust separation conditions, and rapidity of separation. In the same time, a serious drawback of CE is low reproducibility of migration times and peak areas. However, this problem can be resolved through coating the capillary surface with modifiers. Modification of the capillary surface makes it possible to control the magnitude and direction of electroosmotic flow (EOF) and affords a decrease in adsorption of compounds and background electrolyte (BGE) on the inner capillary surface [1]. The modifiers can be classified into two groups, those that alter the EOF directions and the EOF suppressers, and their application can be done either by chemical modification of the inner surface of a capillary (e.g., with polyacrylamide [2, 3]) or by dynamic coating, with the latter option being more simple and convenient.

The cationic components of the BGE usually having a number of binding centers can efficiently interact with the capillary wall, thus charging the surface and affecting the EOF. To prevent that, various cationic polymers are used in the CE that physically adsorb to the capillary surface. They include ionenes [4, 5], quaternary ammonium bases, chitosan [6], and other natural cationic polymers [7, 8]. However, such coatings often are of low durability and thus compromise reproducibility. To enhance stability, bilayer or multilayer coatings can be introduced, for example, by means of successive application of two solutions: a polycationic one and then a polyanionic one [9, 10]. The coatings of this kind can improve separation of proteins, carbohydrates, pharmaceuticals, and their enantiomers by capillary electrophoresis. The first report of the technique of dynamic coating of the capillaries with multiple layers of polyelectrolytes including ionene is due to Katayama et al. The coating can be applied simply by flushing the capillary with a solution of positively

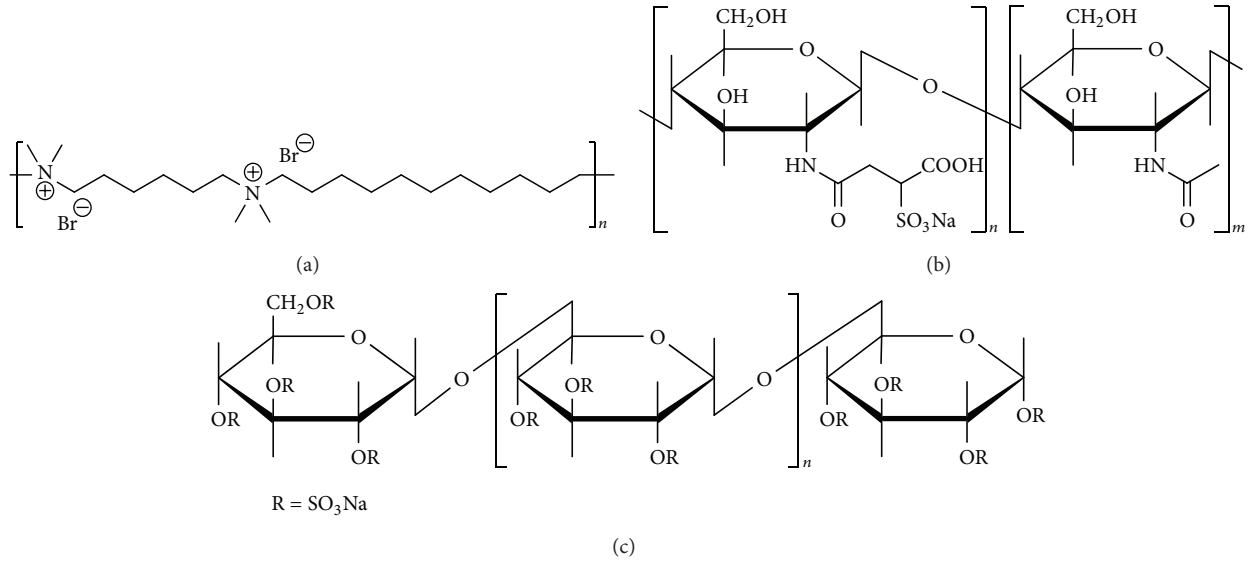


Figure 1: Structures of (a) 6,10-ionene, (b) N-(3-sulfo-3-carboxy)-propionylchitosan, and (c) dextran sulfate.

charged polybrene (hexadimethrine bromide) followed by a solution of negatively charged dextran sulfate (DS) polyelectrolyte [11]. With the coated capillaries, highly reproducible values of the EOF and the plateau height of free drug can be obtained [12].

In the 21st century, growing urbanization has made certain diseases very common, and some of them are even considered now as a new norm. Among them is allergic rhinitis, a disease highly prevalent in the metropolitan areas. While sounding rather innocuous, it can complicate the everyday lives of many persons, such as business people. Common therapies for the allergic rhinitis include α-adrenergic agonists, H_1-blockers, and antiseptics. The aim of the present study was to prepare and assess a capillary for rapid, efficient, and reproducible separation and determination of the above drugs in the biological fluids as a mean of monitoring the patient's condition. We introduce the new multiple layer capillary coatings consisting of polycationic 6,10-ionene and polyanionic N-(3-sulfo-3-carboxy)-propionylchitosan (SCPC) and dextran sulfate (DS) (Figure 1). Atenolol, pindolol, nadolol (β-blockers), tetrahydrozoline (α-adrenergic agonist), terbutaline (β-2-agonist), hydroxyzine, orphenadrine (M-cholinoreceptor blockers), doxylamine, carbinoxamine, and chlorpheniramine (H_1-blockers) were taken as model compounds (Figure 2).

2. Experimental

2.1. Chemicals and Materials. Pindolol, atenolol, nadolol, tetrahydrozoline, terbutaline, and hydroxyzine were purchased from Sigma-Aldrich (USA), and their samples were diluted with deionized water to a concentration of 100 μg/mL. Methyl ethyl ketone (3% water solution) obtained from the Chair of Analytical Chemistry (MSU, Russia) and deionized water itself were used as EOF markers. Sodium citrate, potassium phosphate trihydrate, dipotassium phosphate, and sodium hydroxide for background buffer preparation were purchased from Reachem (Moscow, Russia).

6,10-Ionene was obtained from the Division of Analytical Chemistry (MSU, Russia). N-(3-Sulfo-3-carboxy)-propionylchitosan (average molecular weight 5 kDa, degree of deacetylation 92%) was provided by Dr. V. P. Varlaamov. Dextran sulfate was purchased from Sigma-Aldrich (USA). A 37.8 cm × 50 μm i.d. fused silica capillary (effective length 29.3 cm) used in this study was obtained from Polymicro Technologies (Phoenix, USA).

2.2. Apparatus. The CE analyses were conducted with a Kapel-105M instrument (Lumex, Saint Petersburg, Russia) equipped with an autosampler and a photometric UV detector (λ = 235 and 270 nm). All CE operations were controlled by Elforan software. Migration time, separation efficiency, peak resolution, selectivity, and electrophoretic mobilities were calculated.

The capillaries were thermostated at 20°C using a liquid coolant, and the samples were injected at a pressure of 1000 mbar over 30 s. Electrophoresis was performed at an applied voltage of 8–15 kV.

All solutions were prepared in deionized water under pH control with the AV-74 ionometer and then degassed in an ultrasonic bath (Sapfir, Moscow). The samples were stored at 4°C.

The salts for preparation of the running buffer were diluted in deionized water at a concentration of 25 and 50 mM. Prior to the CE analyses, all buffers were filtered through the 0.22 μm Durapore PVDF membrane filters (Millipore, France). To prepare the background electrolytes, modifiers were weighed and dissolved in running buffers at concentrations of 0.1 and 1.5%.

2.3. Layer-by-Layer Coating Protocol. Bare fused silica capillary was successively rinsed with deionized water, 1 M NaOH,

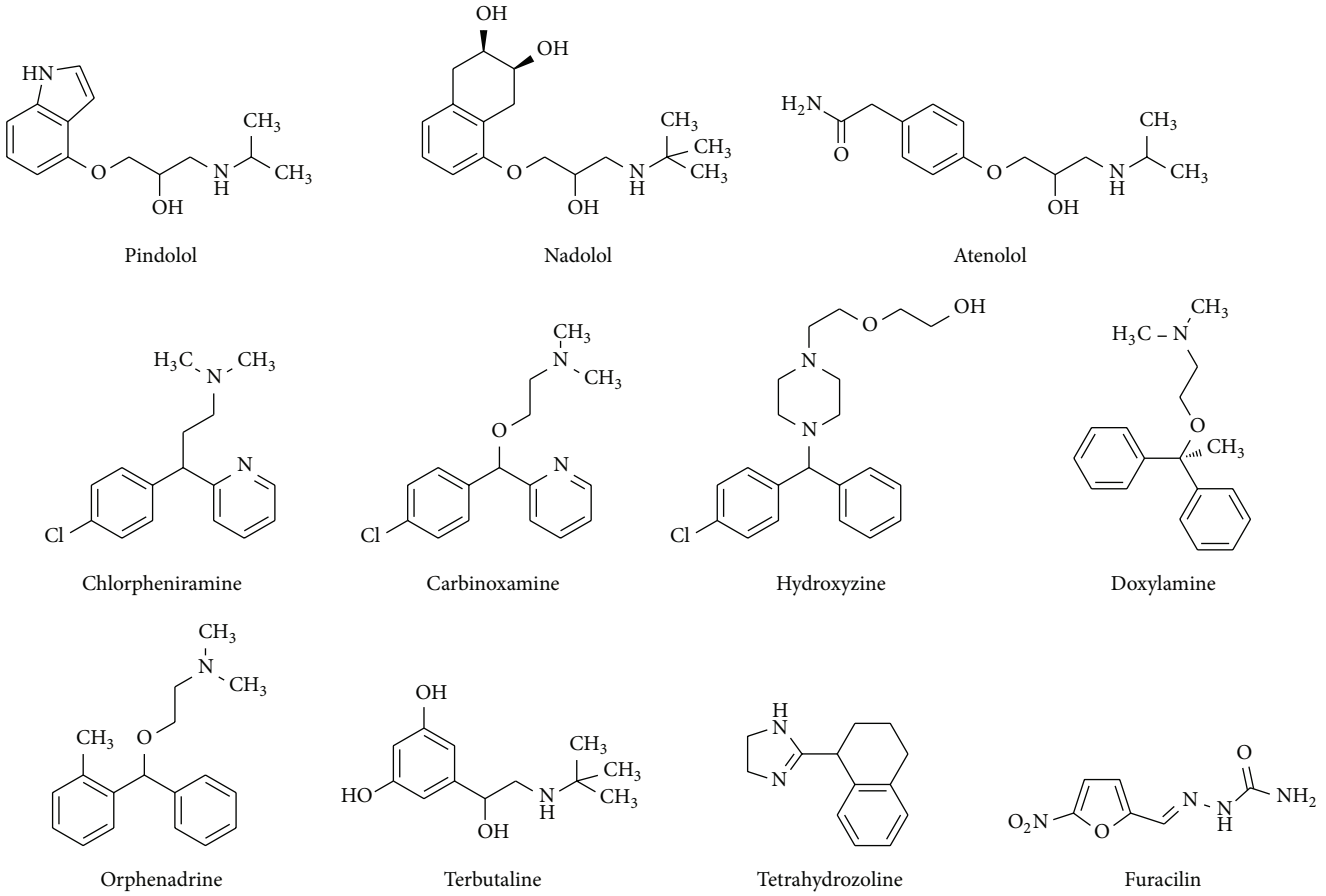


Figure 2: Structures of drugs studied.

and again with water for 10 minutes each. After preconditioning, the capillary was rinsed with a solution of 6,10-ionene (2 mg mL^{-1}) for 20 min at 1000 mbar. Then, the tips of the capillaries were kept overnight in deionized water. On the next day, the capillaries were rinsed with a polysaccharide solution (1 mg/mL) for 40 min and once again left overnight in water.

Before starting an experiment, a fresh capillary was rinsed with water for 10 min and with BGE (25 mM citric buffer at pH 6.5) for 10–30 min. Between any run, the capillary was rinsed with distilled water for 1 min and then with BGE for 2 min. BGE was refreshed every 4-5 analyses.

3. Results and Discussion

3.1. Development of the Method

3.1.1. Selection of the Modifiers. The separation of compounds by means of CE is strongly affected by adsorption on the bare fused silica capillaries, which is due to the electrostatic interactions and hydrogen bonding of the analytes with the silanol groups of the capillary surface. Adsorption results in deterioration of reproducibility and separation performance. Several approaches to overcome this problem have been explored; among them are various coating procedures [8, 11].

Poly (diallyldimethylammonium) chloride is a polycation most commonly used for multilayer coating of capillaries; it serves as a popular test case for studying the stability issues of the coatings. Other promising though less studied polycations are ionenes. Among them, polybrenes have been reported in the literature as capillary coatings. We believe that such compounds can contribute to the expansion of the range of applicable ionenes. In view of that, we employed 6,10-ionene for capillary modification.

Also, polysaccharides are widely used. Capillary coatings based on charged polysaccharides ensure higher reproducibility of migration times compared to the neutral coating techniques. Furthermore, such coatings are mostly devoid of solubility related problems. Negatively charged polysaccharides are used more universally than the neutral ones. In particular, Park et al. have synthesized highly sulfated cyclosophoraoses [13], the sulfated derivatives of chiral unbranched cyclic β-(1 → 2)-D-glucans, and successfully used them to separate five basic chiral drugs (arterenol, atenolol, isoproterenol, propranolol, and metoprolol). The seanionic derivatives enabled better resolution than the original cyclosophoraoses and were therefore recommended for separation of basic drugs. Nishi [14] has compared the performance of the two types of polysaccharides, the neutral dextran and dextrin and the charged chondroitin sulfate C and chondroitin sulfate A, in separation of some basic drugs. Again, anionic polysaccharides provided higher separation efficiency. The most interesting results were obtained with

heparin [15, 16], chondroitin sulfates (A, B, C) [17], colominic acid [18], dextran sulfate [19, 20], and SCPC [6].

Polycations form stable complexes with anionic polyelectrolyte polysaccharides. This behavior can be utilized in the creation of stable capillary coatings, which has been already demonstrated in several studies. Thus, stable and efficient capillary coating with polybrene and dextran sulfate has been obtained [12, 21]. Additional particular attractiveness of anionic polysaccharides is due to their enantiorecognition ability.

Previously, we have effected CE separation of the enantiomers of pindolol, atenolol, nadolol, fluoxetine, chlorcyclizine, hydroxyzine, orphenadrine, terbutaline, and tetrahydrozoline in presence of SCPC in BGE. For enantiomeric separation of fluoxetine, tetrahydrozoline, doxylamine, carbinoxamine, chlorpheniramine, and orphenadrine, we also used an additive of DS in BGE [22]. Comparison of the separation efficiency between original bare fused silica capillaries and modified capillaries would be of high interest.

In view of the foregoing, the present study reports two especially promising modifications of the fused silica capillaries: with N-(3-sulfo-3-carboxy)-propionylchitosan (SCPC) and with dextran sulfate (DS).

3.1.2. Verification of the Capillary Surface Modification. Two coated capillaries were prepared. After initial treatment of a fused silica capillary with 6,10-ionene, one was further coated with N-(3-sulfo-3-carboxy)-propionylchitosan (SCPC capillary) and the other with dextran sulfate (DS capillary). Capillary with four coating layers was also prepared. It will be discussed separately in another part.

Properties of the capillaries were examined after each act of coating by monitoring the EOF direction and value. In the fused silica capillaries, EOF is directed towards the cathode and, accordingly, transfers positive charge (normal polarity mode). After coating with 6,10-ionene, the peak due to the EOF marker was identified in the reversed polarity mode, which agrees with the expected inversion in surface charging (negative to positive). However, the reproducibility of the electroosmotic mobility was less than desired (s_r = 0.09, n = 3) and, moreover, the EOF strength itself was low ($\mu_{EOF} = -8.77 \times 10^{-5}\,cm^2 \cdot V^{-1} \cdot s^{-1}$). After the second modification step, that is, coating with polysaccharides, EOF was once again detected in the normal polarity mode. This observation confirms the adsorption of the polysaccharides on the surface modified with 6,10-ionene. The electroosmotic mobility value for the SCPC capillary and the DS one was $1.24 \cdot 10^{-4}$ and $2.00 \cdot 10^{-4}\,cm^2 \cdot V^{-1} \cdot s^{-1}$, respectively ($s_r$ = 0.08 and 0.05; n = 3).

3.1.3. Selection of the Buffer pH and Composition. In this study, we used a buffer with pH 6.5. Each of the test compounds contains a nitrogen atom, which can be positively charged at pH 4–7. In our preliminary enantioseparation investigations, where we studied some test compounds on a fused silica capillary in presence of DS, the highest efficiency was obtained in the pH range of 6.5–6.8 while at pH above 6 or below 7 none of the compounds were identifiable.

We have tested the phosphate and the citrate buffer known for high buffer capacity in the pH range of interest. With the phosphate buffer, the model compounds migrated in less than 2 min and no separation was achieved even up to buffer concentration of 50 mM. This led us to select the 25 mM citrate buffer, with which we had good experience in the study of capillary modification with citrate-stabilized gold nanoparticles [20]. This buffer afforded resolution of the model compounds within 3–6 min with sufficient quality (R_s = 0.7–10.3). The concentration of 25 mM was found to provide the optimal trade-off between the resolution, which suffers from the increase in the electrophoretic mobility at lower buffer concentrations and the duration of the analyses. Note that apart from the decreased mobility and hence tedious analytical work, higher buffer concentrations can bring about the Joule heating issues.

3.1.4. Selection of the Working Voltage. Trial separations of the test compounds were performed under the applied voltage of 8, 15, and 25 kV. Under 25 kV, the mobility of the test compounds was exceedingly high, which precluded any possible separation. Voltage reduction to 8 kV resulted in the increase in the separation time to 12 min. Yet, for some of the test compounds, complete separation has not occurred. Under the compromise 15 kV conditions, we achieved separation of the same compounds as under 8 kV but with considerable time saving. Thus, we were conducting subsequent experiments under 15 kV.

3.2. Analysis of the Pharmaceuticals. Initially, the model compounds were analyzed using a conventional fused silica capillary. The order of migration of the model compounds was as follows: tetrahydrozoline, pindolol, atenolol, terbutaline, nadolol, and finally hydroxyzine. Electrophoretic mobilities were rather high (up to $7.02 \cdot 10^{-4}\,cm^2 \cdot V^{-1} \cdot s^{-1}$). The mixture of tetrahydrozoline, pindolol, terbutaline, nadolol, and hydroxyzine was separated within 5 minutes (Figure 3). This figure shows the low height of peaks and bad peak resolution (not to baseline). Stability of the fused silica capillary was unsatisfactory and so was the reproducibility of the electroosmotic mobility and analyte mobility (s_r = 0.82 and 0.57, resp.; n = 3). Within three days, the efficiency of the capillary dropped by 30% and more. However, as we show below, the separation performance was improved through the use of the coated capillaries.

3.2.1. SCPC Capillary. The SCPC capillary was rinsed with BGE for 20 min for equilibration. The order of migration for the model compounds with the SCPC capillary was as follows: tetrahydrozoline, atenolol, pindolol, nadolol, terbutaline, and hydroxyzine. The migration rate depends on the value of the positive charge at the nitrogen atom, as well as the size and structure of the analytes. Higher charge and smaller molecular size result in higher mobility and, correspondingly, lower migration time of the substance. In addition, mobility can be influenced by the interaction of the analytes with SCPC at the inner capillary surface. Tetrahydrozoline, pindolol, nadolol, terbutaline, and hydroxyzine were separated

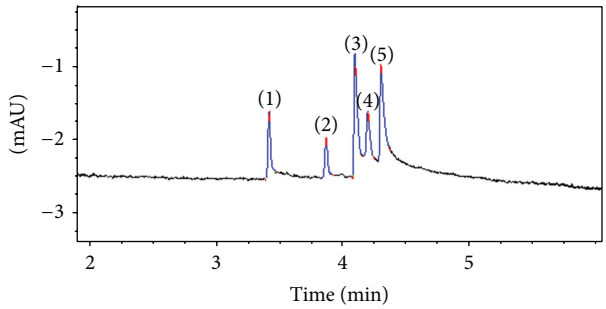


Figure 3: Separation of (1) tetrahydrozoline, (2) pindolol, (3) terbutaline, (4) nadolol, and (5) hydroxyzine by capillary zone electrophoresis with spectrophotometric detection (λ = 235 nm) on the fused silica capillary. CE conditions: $L_{tot/eff}$ 38.7/29.3 cm; BGE solution: 25 mM citric buffer at pH 6.5; voltage 15 kV, hydrodynamic injection for 30 s with 10 mbar.

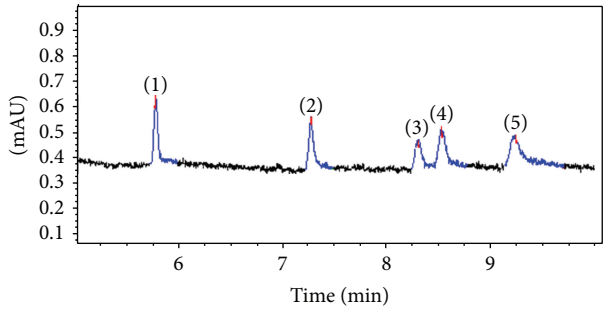


Figure 4: Separation of (1) tetrahydrozoline, (2) pindolol, (3) nadolol, (4) terbutaline, and (5) hydroxyzine on the SCPC capillary. Conditions as in Figure 3.

within 10 min with an efficiency of up to 125000 TP (Figure 4, Table 1).

The SCPC levels were varied in the range of 0 to 1.08%. The mobility of the compounds grew upon the increase in the SCPC concentration from 0.0 to 0.15% while beyond 0.15% it started to decrease back. This is likely due to the CS migration in the opposite direction with respect to the EOF. The highest efficiency for 4 of the 6 compounds studied (up to 170000) was reached at the upper limit of the tested SCPC concentrations range (Table 2).

3.2.2. DS Capillary. The capillary was rinsed with BGE for 10 min for equilibration. The migration order of model compounds altered again: pindolol and terbutaline were detected before atenolol and nadolol, respectively.

Migration times for this capillary were lower than those for the SCPC capillary but higher than those with a fused silica capillary. Tetrahydrozoline, pindolol, nadolol, terbutaline, and hydroxyzine were separated on a DS capillary within 7 min with higher efficiency (up to 200000 TP) and comparable or, sometimes, even higher selectivity (Figure 5, Table 3).

For an extended set of test compounds, the migration order was as follows: tetrahydrozoline, pindolol, atenolol,

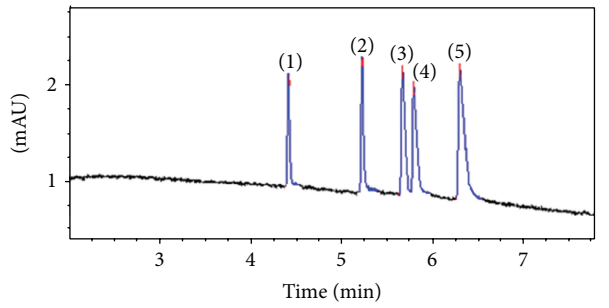


Figure 5: Separation of (1) tetrahydrozoline, (2) pindolol, (3) terbutaline, (4) nadolol, and (5) hydroxyzine on the DS capillary. Conditions as in Figure 3.

terbutaline, nadolol, orphenadrine, doxylamine, carbinoxamine, hydroxyzine, and chlorpheniramine.

The effect of the DS concentration in BGE on the mobility of compounds and on the efficiency of the capillary was investigated in the range of 0 to 1.5%. Similar to the SCPC case, the mobilities of the compounds showed initial increase up to SCPC concentration of 0.1% while beyond this level the trend was inverted. The effect of DS on the efficiency of the capillary was uneven over the reported range of compounds. However, addition of the DS to the citrate buffer always yielded higher efficiency compared to the neat buffer.

In general, the DS coating was found to be highly stable and efficient against analyte adsorption. It afforded better reproducibility and performance compared to the standard bare fused silica capillary.

3.2.3. Comparison of the Modified Capillaries and Their Advantages over Fused Silica Capillaries. As was shown above, modification of the capillary surface improved its chemical stability and reproducibility of the separation results compared to a fused silica capillary. Migration times of the model compounds became longer (with citrate running buffer as BGE), which may be explained by incomplete shielding of the cationic centers of 6,10-ionene by the sulfo groups of the polysaccharides. This implies lower integral negative charge on the inner capillary surface and consequential reduction in the EOF rate.

The changes in the order of migration of the model compounds may be due to the CH_2–OH functions in the SCPC. For example, terbutaline can develop stronger hydrogen-bonding interactions than nadolol as its hydroxy groups are attached to benzene rather than a cyclohexane ring. As a result, with SCPS, the migration time of terbutaline becomes longer than that of nadolol. The behavior of atenolol-pindolol pair, however, is likely to be governed by some different types of interactions or more subtle effects. Indeed, atenolol comes out earlier from the SCPC capillary (but not from the DS one) despite being more polar and capable of more efficient hydrogen bonding (an amide group versus a pyrrole ring in pindolol). Yet another advantage of the modified capillaries is higher separation efficiency, especially in the case of DS. We observe a 2-3-fold increase in separation efficiency peaking at 430500 TP. Furthermore, both capillaries provide improved

Table 1: Separation of tetrahydrozoline, pindolol, nadolol, terbutaline, and hydroxyzine on the SCPC and DScapillaries. Conditions as in Figure 3.

	Compound	t_m, min	α	R_s	N, TP
SCPC capillary	Tetrahydrozoline	5.78	1.26	10.7	125810
	Pindolol	7.27	1.14	7.3	102890
	Nadolol	8.30	1.02	1.4	90370
	Terbutaline	8.53	1.08	3.0	65640
	Hydroxyzine	9.24			28690
DS capillary	Tetrahydrozoline	4.42	1.24	7.4	197970
	Pindolol	5.22	1.08	3.2	166930
	Terbutaline	5.67	1.02	0.8	115310
	Nadolol	5.79	1.09	2.1	97310
	Hydroxyzine	6.30			52300

Table 2: Effect of SCPC concentration in BGE on the migration times of analytes and efficiency of SCPC capillary (λ = 235, 270 nm). Other conditions as in Figure 3.

Compound	SCPC concentration, %							
	0		0.10		0.15		1.08	
	t_m, min	N, TP	t_m, min	N, TP	t_m, min	N, TP	t_m, min	N, TP
Tetrahydrozoline	5.5	79800	3.1	98740	2.8	79970	3.5	141280
Athenolol	6.7	40370	3.5	34350	3.6	35280	3.8	60250
Pindolol	7.0	76260	3.6	136260	3.5	151600	3.9	57760
Nadolol	8.0	49450	3.7	80180	3.7	93880	4.0	170980
Terbutaline	8.2	66460	3.8	68690	3.8	70310	4.0	141230
Hydroxyzine	8.9	41700	3.8	81250	3.8	72510	4.2	8480

reproducibility of the compound mobilities with citrate buffer as BGE (s_r = 0.001–0.049 and 0.001–0.011 for the SCPC and the DS capillary, resp.; n = 3). Apparently, this is in connection with the stability of the capillary surface. Note that when the polysaccharides were used only as chiral selectors (CS) without surface modification, capillaries had to be replaced every 2-3 days [9, 16] while the modified capillaries retained their properties for at least 4 weeks (Table 3). In general, the DS capillary was found to be more promising for the purposes of separation and enantioseparation of various pharmaceuticals and for other related tasks.

For the successful combination of 6,10-ionene and DS, we have further investigated the effect of repeated multilayer capillary coatings. A capillary modified with four alternating coating layers, two of 6,10-ionene and two of DS, was prepared. This modification was found to increase the electrophoretic mobility of the model compounds. Tetrahydrozoline, pindolol, doxylamine, chlorpheniramine, orphenadrine, nadolol, and hydroxyzine were separated within 7 min (U = 8 kV) with an efficiency of up to 225000 TP.

3.3. Ability of Modified Capillaries for Enantiorecognition. As was expected, in case of SCPC capillary with chiral selector- (CS-) free BGE, no enantioseparation was observed (as for other such capillaries). Enantiomers of atenolol and terbutaline (R_s = 0.6 and 0.8, resp.) were separated with BGE containing 0.1% SCPC. However, the capillary properties were found to change over time, which ultimately made enantioseparation impossible.

Enantiomers of tetrahydrozoline and atenolol were separated on a DS capillary at a rather low DS concentration of 0.1% within 3 min (R_s = 1.2 and 0.8 and s_r = 0.03 and 0.05, resp.; n = 3). Enantiomers of terbutaline (R_s = 1.0, s_r = 0.013; n = 3), orphenadrine (R_s = 1.0, s_r = 0.001; n = 3), doxylamine (R_s = 0.8, s_r = 0.001; n = 3), and chlorpheniramine (R_s = 0.8, s_r = 0.001; n = 3) were separated at 1.5% of DS in BGE within 4 min.

A capillary double modified with 6,10-ionene and DS (four coating layers) provided better resolution of the enantiomers of the model compounds and made it possible to conduct the analyses at lower DS concentrations in BGE. For instance, enantiomers of tetrahydrozoline (R_s = 1.4), doxylamine (R_s = 1.0), carbinoxamine (R_s = 0.9), and hydroxyzine (R_s = 0.8) were separated with 1.08% DS in BGE (Figure 6). Note that with a capillary with two coating layers, we did not observe separation of the latter two compounds even at 1.5% DS in BGE; similarly, the enantiomers of hydroxyzine were not separated in which a bare fused silica capillary was used and DS was added in BGE [23]. So, it is another advantage of coated capillaries over the bare fused silica capillaries.

However, it has to be said that the coated capillaries are far from perfect for the purposes of enantioseparation. The major problems include the less than preparative scale of the method and insufficient peak resolution. The latter drawback is likely due to a combination of two mechanisms: the HPLC-like mechanism on the capillary surface (its contribution is low since the amount of CS on the surface is rather small) and

Table 3: Comparison stability of the coatings in terms of electroosmotic mobility for the coated capillaries.

SCPC capillary						
EO mobility $\cdot 10^4$, $cm^2 \cdot V^{-1} \cdot s^{-1}$	RSD $n = 3$	EO mobility $\cdot 10^4$, $cm^2 \cdot V^{-1} \cdot s^{-1}$	RSD $n = 3$	EO mobility $\cdot 10^4$, $cm^2 \cdot V^{-1} \cdot s^{-1}$	RSD $n = 3$	Variation of EO mobility, RSD
Before treatment		After treatment (2 weeks)		After treatment (1 month)		Over time of life
1.24	0.08	1.20	0.04	1.15	0.06	0.07
DS capillary						
EO mobility $\cdot 10^4$, $cm^2 \cdot V^{-1} \cdot s^{-1}$	RSD $n = 3$	EO mobility $\cdot 10^4$, $cm^2 \cdot V^{-1} \cdot s^{-1}$	RSD $n = 3$	EO mobility $\cdot 10^4$, $cm^2 \cdot V^{-1} \cdot s^{-1}$	RSD $n = 3$	Variation of EO mobility, RSD
Before treatment		After treatment (1 week)		After treatment (5 months)		Over time of life
2.00	0.05	2.00	0.03	1.98	0.04	0.01

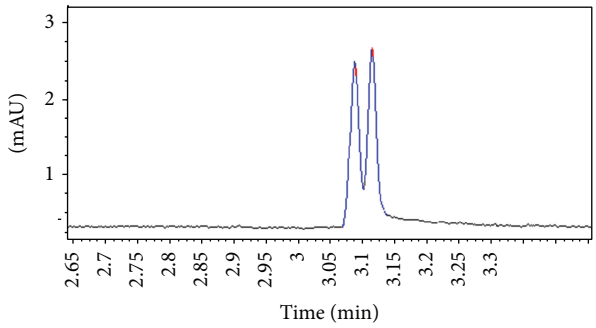


Figure 6: Separation of enantiomers of tetrahydrozoline in the capillary with four coating layers (6,10-ionene and DS). CE conditions: $L_{tot/eff}$ 38.7/29.3 cm; BGE solution: 1.08% DS in 25 mM citric buffer at pH 6.5; voltage 15 kV and hydrodynamic injection for 30 s with 10 mbar, λ = 235 nm.

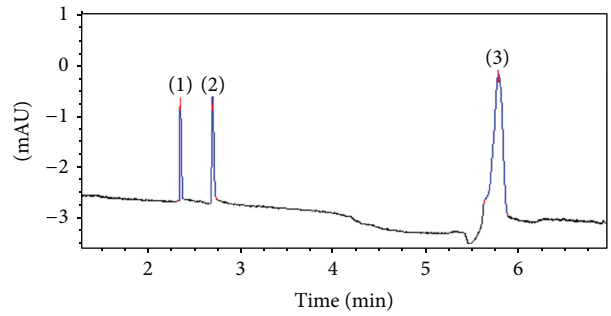


Figure 7: Separation of (1) tetrahydrozoline, (2) carbinoxamine, (3) and furacilin in human urea by capillary zone electrophoresis with spectrophotometric detection (λ = 235 nm) on the DS capillary. CE conditions: $L_{tot/eff}$ 38.7/29.3 cm; BGE solution: 25 mM citric buffer at pH 6.5; voltage 15 kV and hydrodynamic injection for 30 s with 10 mbar.

the CE mechanism in the bulk of the solution that involves the CS additives to the BGE. In some cases, the order of migration differs for these two concurrent mechanisms, and their interplay consequently results in lower peak resolution compared to the CE-only process in the pristine fused silica capillaries.

3.4. Application of the DS Capillary to the Analysis of Human Urea. The capillary modified with 6,10-ionene and dextran sulfate was used for separation of drugs for the treatment of the allergic rhinitis. Tetrahydrozoline (alpha-adrenergic agonist), carbinoxamine (H_1-blocker), and furacilin (antiseptic) (Figure 1) were separated within 6 min with an efficiency of up to 78000 TP (Figure 7). The applied voltage was 15 kV and the detection wavelength was 235 nm. The quantitative accuracy was independently verified by HPLC.

Sample preparation included tenfold dilution of the test sample with BGE and centrifugation for 5 min (10000 r.p.m.). Identification was carried out by means of the standard addition method. The results and the metrological parameters are presented in Table 4.

The control HPLC identification was performed using a Shimadzu HPLC system equipped with an SPD-M20A photodiode array detector and an LC-20AB binary pump. We used commercially available Mightysil C-18 as the stationary phase and 50/50 mixture of acetonitrile and 0.1% water solution of the phosphoric acid as an eluent. As shown in Table 4, there is a good agreement between the HPLC and the CE results.

4. Conclusions

The present study has demonstrated that 6,10-ionene and dextran sulfate can be recommended for modifying the CE capillaries by forming multilayer coatings on the inner surface. In addition, dextran sulfate can be used as a chiral selector as well. For the capillaries thus coated, we report the optimal conditions for identification and separation of tetrahydrozoline (alpha-adrenergic agonist), carbinoxamine (H_1-blocker), and furacilin in human urea. The DS-modified capillaries are also suitable for separation of other nitrogen-containing compounds and their enantiomers.

Table 4: CE and HPLC determination of tetrahydrozoline, carbinoxamine, and furacilin in human urea. CE conditions as in Figure 6 and, for sample preparation, see Results and Discussion.

	Contention, $\mu g\,mL^{-1}$		$c_{,min}$, $\mu g\,mL^{-1}$	linearity	S_r ($n = 3$)	Regression equation	R^2
CE	Tetrahydrozoline	5.3 ± 0.8	1.0	2-140	0.07	$y = (0.167 \pm 0.004)\,x$	0.996
	Carbinoxamine	6.6 ± 0.5	0.8	2-140	0.03	$y = (0.256 \pm 0.008)\,x$	0.997
	Furacilin	0.9 ± 0.2	0.6	2-140	0.10	$y = (1.696 \pm 0.615)\,x + (12.473 \pm 0.416)$	0.997
HPLC	Tetrahydrozoline	4.9 ± 1.7	0.5	2-100	0.08	$y = (20.6 \pm 0.9)\,x$	0.999
	Carbinoxamine	6.2 ± 0.4	0.5	2-100	0.05	$y = (7.8 \pm 0.2)\,x$	0.999
	Furacilin	0.6 ± 0.1	0.5	2-100	0.08	$y = (28.5 \pm 0.6)\,x + (1.9 \pm 0.3)$	0.999

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was financially supported by the Russian Foundation for Basic Research (Project no. 12-03-00405a) and the Ministry of Education and Science of the Russian Federation (no. 8245).

References

[1] J. Znaleziona, J. Petr, R. Knob, V. Maier, and J. Ševčík, "Dynamic coating agents in CE," *Chromatographia*, vol. 67, no. 1, pp. S5–S12, 2008.

[2] J. Horvath and V. Dolnik, "Polymer coatings for capillary electrophoresis," *Electrophoresis*, vol. 22, pp. 644–655, 2001.

[3] T. M. Oswald and T. J. Ward, "Enantioseparations with the macrocyclic antibiotic ristocetin A using a countercurrent process in CE," *Chirality*, vol. 11, p. 663, 1999.

[4] W. Jaeger, J. Bohrisch, and A. Laschewsky, "Synthetic polymers with quaternary nitrogen atoms-Synthesis and structure of the most used type of cationic polyelectrolytes," *Progress in Polymer Science*, vol. 35, no. 5, pp. 511–577, 2010.

[5] Z. Jiang, J. Kang, D. Bischoff, B. Biser, R. D. Sussmuth, and V. Schurig, "Avoparcin, a new macrocyclic antibiotic chiral run buffer additive for capillary electrophoresis," *Electrophoresis*, vol. 20, no. 12, pp. 2438–3457, 1999.

[6] N. Y. Budanova, E. N. Shapovalova, and O. A. Shpigun, "Looking into the possibility of using of chitosan in capillary electrophoresis," *Moscow University Chemistry Bulletin*, vol. 47, no. 3, pp. 177–181, 2006.

[7] T. W. Graul and J. B. Schlenoff, "Capillaries modified by polyelectrolyte multilayers for electrophoretic separations," *Analytical Chemistry*, vol. 71, no. 18, pp. 4007–4013, 1999.

[8] R. Nehmé, C. Perrin, H. Cottet, M.-D. Blanchin, and H. Fabre, "Stability of capillaries coated with highly charged polyelectrolyte monolayers and multilayers under various analytical conditions—application to protein analysis," *Journal of Chromatography A*, vol. 1218, no. 22, pp. 3537–3544, 2011.

[9] J. Janssens, R. Chevigne, and P. Louis, "Capillary electrophoresis method using initialized capillary and polyanion-containing buffer and chemical kit therefore," U.S. Patent 5,611,903, Mart, 1997.

[10] G. Vanhoenacker, F. de l'Escaille, D. de Keukeleire, and P. Sandra, "Dynamic coating for fast and reproducible determination of basic drugs by capillary electrophoresis with diode-array detection and mass spectrometry," *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, vol. 799, no. 2, pp. 323–330, 2004.

[11] H. Katayama, Y. Ishihama, and N. Asakawa, "Stable capillary coating with successive multiple ionic polymer layers," *Analytical Chemistry*, vol. 70, no. 11, pp. 2254–2260, 1998.

[12] C. Liu and J. Kang, "Improved capillary electrophoresis frontal analysis by dynamically coating the capillary with polyelectrolyte multilayers," *Journal of Chromatography A*, vol. 1238, pp. 146–151, 2012.

[13] H. Park, S. Lee, S. Kang, Y. J. Jung, and S. H. Jung, "Enantioseparation using sulfated cyclosophoraoses as a novel chiral additive in capillary electrophoresis," *Electrophoresis*, vol. 25, no. 16, pp. 2671–2674, 2004.

[14] H. Nishi, "Enantiomer separation of basic drugs by capillary electrophoresis using ionic and neutral polysaccharides as chiral selectors," *Journal of Chromatography A*, vol. 735, no. 1-2, pp. 345–351, 1996.

[15] A. M. Stalcup and N. M. Agyel, "Heparin: a chiral mobile-phase additive for capillary zone electrophoresis," *Analytical Chemistry*, vol. 66, no. 19, pp. 3054–3059, 1994.

[16] N. M. Agyei, K. H. Gahm, and A. M. Stalcup, "Chiral separations using heparin and dextran sulfate in capillary zone electrophoresis," *Analytica Chimica Acta*, vol. 307, no. 2-3, pp. 185–191, 1995.

[17] H. Nishi, K. Nakamura, H. Nakai, and T. Sato, "Enantiomeric separation of drugs by mucopolysaccharide-mediated electrokinetic chromatography," *Analytical Chemistry*, vol. 67, no. 14, pp. 2334–2341, 1995.

[18] Y. Du, A. Taga, S. Suzuki, W. Liu, and S. Honda, "Colominic acid: a novel chiral selector for capillary electrophoresis of basic drugs," *Journal of Chromatography A*, vol. 962, no. 1-2, pp. 221–231, 2002.

[19] K. W. Phinney and L. C. Sander, "Enantioselective separations in capillary electrophoresis with dextran sulfate as the chiral selector," *Analytical and Bioanalytical Chemistry*, vol. 375, no. 6, pp. 763–768, 2003.

[20] A. N. Mikhalyuk, E. N. Shapovalova, A. G. Maguga, O. A. Shpigun, and P. G. Rudakovskaja, "Electrophoretic separation of nitrogen-containing drugs using silica capillaries modified with citrate-stabilized gold nanoparticles, 6,10-ionene, N-(3-sulfo,3-carboxy)-propionylchitosan and dextran sulfate," *Moscow University Chemistry Bulletin*, vol. 68, no. 5, pp. 219–222, 2013.

[21] R. Nehmé, C. Perrin, H. Cottet, M. D. Blanchin, and H. Fabre, "Influence of polyelectrolyte coating conditions on capillary coating stability and separation efficiency in capillary electrophoresis," *Electrophoresis*, vol. 29, no. 14, pp. 3013–3023, 2008.

[22] N. Budanova, E. Shapovalova, S. Lopatin, V. Varlamov, and O. Shpigun, "N-(3-sulfo, 3-carboxy)-propionylchitosan as new chiral selector for enantioresolution of basic drugs by capillary electrophoresis," *Chromatographia*, vol. 59, no. 11-12, pp. 709–713, 2004.

[23] N. Budanova, D. Shamshurin, and E. Shapovalova, "High molecular dextran sulfate as chiral selector in CE," in *Proceedings of the 10th International Conference on Theoretical Problems of Surface Chemistry, Adsorption and Chromatography*, A. A. Fomkin and G. A. Petuhova, Eds., pp. 386–391, Granici, Moscow, Russia, April 2006.

Hindawi Publishing Corporation
Journal of Chemistry
Volume 2014, Article ID 367236, 9 pages
http://dx.doi.org/10.1155/2014/367236

Research Article

Purification of Anthocyanins with o-Dihydroxy Arrangement by Sorption in Cationic Resins Charged with Fe(III)

Araceli Castañeda-Ovando, Carlos Andrés Galán-Vidal, Elizabeth Contreras-López, and Ma. Elena Páez-Hernández

Academic Area of Chemistry, Autonomous University of Hidalgo State, 42184 Mineral de la Reforma, HGO, Mexico

Correspondence should be addressed to Araceli Castañeda-Ovando; ovandoa@uaeh.edu.mx

Received 26 May 2014; Revised 7 August 2014; Accepted 8 August 2014; Published 11 September 2014

Academic Editor: Hasan Uslu

Copyright © 2014 Araceli Castañeda-Ovando et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

In the present work, a new purification method of anthocyanins with o-dihydroxy arrangement is proposed. This method is based on a ligand-exchange mechanism, using a cationic exchange resin loaded with metallic ions in order to increase the affinity of the resin to the anthocyanin(s) with o-dihydroxy arrangement. This method was used to purify the main anthocyanin (cyanidin-3-glucoside; Cy-3-glc) from the anthocyanic methanolic extract of blue corn. The best sorption result was using Fe(III) in its ion form. The purification procedure begins with the formation of a metal-anthocyanin complex (Cy-3-glc-Fe) which was optimal at pH 5, followed by a NaOH 0.1 M elution process in order to eliminate anthocyanins without o-dihydroxy arrangement, sugars, and organic acids. Finally, the pure anthocyanin is obtained by adding HCl 0.1 M which breaks the metal-anthocyanin complex.

1. Introduction

Anthocyanins are the most important pigments on vascular plants and are responsible for the bright orange, pink, red, violet, and blue colors in the flowers and fruits of some plants. They are harmless and easy to incorporate into aqueous systems. These characteristics make them interesting to be used as natural hydrosoluble colorants [1]. Another important anthocyanins property is their high antioxidant activity, which plays a significant role in the prevention of neuronal, chronic degenerative, and cardiovascular diseases, such as cancer, arteriosclerosis, and diabetes [2].

The content of these pigments is low. Fresh fruits exhibit a range of 100–5000 μg/g, depending on the species [3]. In cereals such as rice, maize, wheat, and barley, the contents are in the range of 7–3000 μg/g [4].

Recently, these natural compounds have been increasing their economic importance in the industrial scope of the natural colorants field, and more specifically in the anthocyanins field. Nowadays, it is necessary more than ever to count on alternatives that improve the yield of the extraction process and to find simple and economical purification methodologies that can be applied in the industry [5, 6].

Due to the polar nature of anthocyanins, the most used extraction methods currently consist of extractions with aqueous sulfurated solutions or with polar acidified solvents [7]. Methanol and ethanol are the most used solvents in anthocyanins extraction, being the first one the most efficient at 20% [1]. However, the proposed extraction methods are not very selective and a great number of sample components are coextracted, such as sugar or organic acid [8]. Thus, when the extraction process is completed, it is essential to implement purification techniques in order to isolate the anthocyanin(s) of interest.

For the purification of anthocyanins diverse analytical techniques have been proposed, from solid phase extractions (SPE) [9–11] to the use of chromatographic techniques, such as countercurrent chromatography (CCC) [12], medium pressure liquid chromatography (MPLC) [13], and high performance liquid chromatography (HPLC) [14–19].

Usually SPE is carried out in C_{18} or Sephadex cartridges. In this case the anthocyanins are bounded strongly to these adsorbents by their hydroxyl groups. After the adsorption they are separated from other compounds by consecutive increases of the polarity of different solvents [20].

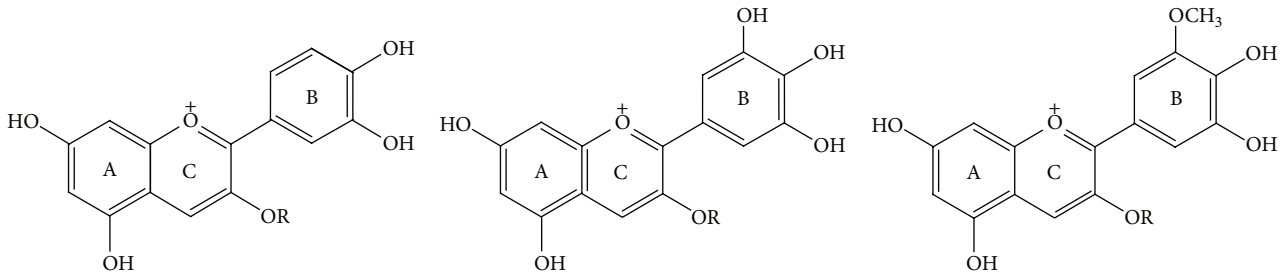


Figure 1: Chemical structures of anthocyanins with o-dihydroxy arrangement; (a) cyanidin (Cy), (b) delphinidin (Dp) y, (c) petunidin (Pt). R = monosaccharide or disaccharide.

CCC and MPLC are used as purification methods with later analysis by HPLC for the structural elucidation, with the advantage that it reduces the separation time and the cost of the mobile phase [1]. HPLC has been reported more frequently as the separation method for anthocyanins. However, it is required to spend a great amount of solvents and time to obtain a significant amount of purified pigment.

In the search of simple and economic purification methodologies, alcohol eluents such as ethanol or propanol are used in the adsorption-desorption of anthocyanins in styrene-divinylbenzene adsorbents. This is one of the purification methods that have recently been explored [5]. The recovery percentage obtained ranged from 96 to 100% of total anthocyanins. Although the use of cationic exchange resins could be an interesting alternative of purification, there are not reports about it. It is probably due to the flavylium ion being such a polar molecule that the resin presents a very strong adsorption.

Considering that the anthocyanins and anthocyanidins with o-dihydroxy arrangement are very good at forming metal complexes [21, 22] (Figure 1), in the present work a simple and economic purification method of anthocyanins with o-dihydroxy arrangement in ring B (Figure 1) is reported. The method is based on the ligand-exchange mechanism on a Fe(III)-loaded cation exchange resin.

2. Materials and Methods

2.1. Reagents. All chemicals were used as received and were analytical grade. Methanol HPLC grade (≥99.9%) was purchased from Mallinckrodt Baker (NJ, USA). HCl (36–38%), NaOH (≥98%, pellets), $FeCl_3{\cdot}6H_2O$ (97%), $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$ (≥98%), $CuSO_4{\cdot}5H_2O$ (≥98%), $CaCl_2$ (≥97%), $MgSO_4{\cdot}7H_2O$ (≥98%), acetic acid (≥99.7%), sodium acetate (≥99%), strong acid cationic resin DOWEX-50W-X8 (H^+ form), H_3BO_3 (≥99.5%), and H_3PO_4 (85%) were purchased from Sigma-Aldrich (MO, USA). Deionized water (18.2 MΩ cm) from a Milli-Q equipment of Millipore (MA, USA) was used to prepare all the solutions. One batch of 10 kg pigmented blue corn was kindly donated by Colegio de Postgraduados (Mexico).

2.2. Apparatus. UV-vis spectra were obtained in a Lambda 40 spectrophotometer from Perkin-Elmer at a scanning speed of 60 nm min^{-1}. HPLC analyses were performed on a Perkin Elmer Series 200 liquid chromatograph from Perkin Elmer (MA, USA), equipped with a UV-vis detector at 515 nm and manual injector (sample volume of 50 μL).

2.3. Extraction Procedure. The crude extract was obtained by stirring 50 g of sample (grounded grain of pigmented corn) with five portions (250 mL each one) of 2% (v/v) HCl-methanol solution at 4°C for 2 h. The raw extract was concentrated under vacuum in a rotary evaporator from Büchi (Buch, Switzerland) at room temperature to reach a final volume of 10 mL. Later on, a liquid-liquid extraction (LLE) was carried out with diethyl ether (2 extractions with 25 mL of solvent each one) to remove lipidic material or liposoluble substances contained in the raw extract [23]. The extract was preserved in methanolic solution for the following experiments.

2.3.1. Preparation of Solutions. For this experiment the following solutions were prepared: HCl (1 and 0.1 mol L^{-1}), NaOH (0.1 and 0.01 mol L^{-1}), Fe^{3+} (0.1 mol L^{-1}, from $FeCl_3{\cdot}6H_2O$), Fe^{2+} (0.1 mol L^{-1}, from $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$), Cu^{2+} (0.1 mol L^{-1}, from $CuSO_4{\cdot}5H_2O$), Ca^{2+} (0.1 mol L^{-1}, from $CaCl_2$), and Mg^{2+} (0.1 mol L^{-1}, from $MgSO_4{\cdot}7H_2O$). All the solutions for the different metals were prepared in HCl 0.1 mol L^{-1}. In order to prepare the solution of the extract at different pH values for the sorption experiments, five replicates of 1 mL of the semipurified extract were taken, each one of these was diluted at 25 mL and then pH was adjusted with a HCl 0.1 M solution from pH 1 to 2, and with a buffer acetate solution from pH 3 to 5. The buffer acetate solution (5×10^{-2} mol L^{-1}, pH 5.0) was prepared from acetic acid and sodium acetate.

2.3.2. Resin Preparation. A strong acid cationic resin DOWEX-50W-X8 (H^+ form) was used for all sorption experiments. The procedure carried out for each one of the metallic ions consisted of the following steps: (1) conditioning of the resin by stirring it with HCl 1 mol L^{-1}

solution during 1 hour with the purpose of having it in its acidic form; (2) washing of the resin with distilled water until residual pH was 7; and (3) conditioning of the resin for each one of the metallic ions (Fe^{3+}, Fe^{2+}, Cu^{2+}, Ca^{2+} and Mg^{2+}), by stirring it with 0.1 mol L^{-1} of the metal solution for 30 minutes in order to load the resin on each one of its cationic forms.

2.3.3. Sorption Experiments. Five resin samples loaded with different ions (Fe^{3+}, Fe^{2+}, Cu^{2+}, Ca^{2+}, and Mg^{2+}) were weighted (0.2 g approximately) and were placed in Pasteur pipettes simulating chromatographic columns. After that, 10 mL of extract solution at different pH values were added, following the next elution steps.

(1) No retained compounds were found on the first fraction obtained. In this stage the same solution served as eluent. A red color in the solution was observed.

(2) Afterwards, 2 mL of NaOH 0.1 mol L^{-1} solution was added as eluent, eluting those compounds that still remained in the column but that had not reacted with the metallic ion (Fe^{3+}, Fe^{2+}, Cu^{2+}, Ca^{2+}, or Mg^{2+}). A violet color was perceived in the resin.

(3) The last eluent added was 4 mL of HCl 0.1 mol L^{-1} solution, collecting all fractions obtained. Absorbance measurements were made in quartz cells with 10-mm path length of Perkin Elmer.

2.3.4. Sorption Experiments at pH 5. According to previous results and research about complex Fe(III)-anthocyanin formation [22], it was decided to perform the same procedure described in Section 2.3.2. While using greater resin and extract amount at pH 5 and the loaded-resin with Fe(III) with the purpose of increasing the concentration of the eluted anthocyanin. An acetates solution was employed as buffer.

A solution containing 10 mL of extract and diluted at 50 mL with acetates buffer (0.05 mol L^{-1}, pH = 5.0) was prepared. The elution procedure followed was the same as the one described in Section 2.3.3, but with modifications in the amount of resin (6.0027 g of dry resin) and loaded only with Fe^{3+} 0.1 mol L^{-1} solution (from $FeCl_3 \cdot 6H_2O$, in HCl 0.1 mol L^{-1}), since Fe(III) was the metal with the best sorption results.

The resin was packed in a chromatographic column (i.d. 2 cm), adding 25 mL of extract solution, following the same elution steps described in Section 2.3.3, adding 12.5 mL of NaOH 0.1 mol L^{-1} solution and 25 mL of HCl 0.1 mol L^{-1}, and collecting all fractions obtained.

The fractions collected at each elution step were analyzed by HPLC according to Castañeda-Ovando et al. [24], using an XTerra column from Waters (250 × 4.6 mm, 4 μm).

2.3.5. Acidity Constants of the Purified Anthocyanin. Determinations at different pH experimental values were carried out in the following fashion: for pH 1 the adjustment was made with HCl 1 mol L^{-1} solution. For the range of pH 2–11, a Britton-Robinson buffer (H_3BO_3 0.1 mol L^{-1}, H_3PO_4 0.1 mol L^{-1} and CH_3COOH 0.1 mol L^{-1}) was used adding NaOH until the required values were obtained. And for pH 12 and 13, the pH was adjusted with NaOH 0.01 and 0.1 mol L^{-1} respectively.

The fractions obtained in the last elution step at pH 5 were collected and 13 aliquots of 1 mL each one were diluted with buffer solution at a pH ranging from 1 to 13. The UV-vis spectra of each solution were obtained in a spectral window of 200–600 nm.

3. Results and Discussion

The purification method of anthocyanins with o-dihydroxy arrangement consisted in loading a cationic exchange resin with different ions, which have been reported as producers of metallic o-dihydroxy anthocyanin complexes, such as Fe^{3+}, Fe^{2+}, Cu^{2+}, Ca^{2+}, and Mg^{2+} [22, 25, 26]. Subsequently a ligand-exchange mechanism with the main anthocyanin present in pigmented corn (cyanidin-3-glucoside) was carried out. This method has been reported mainly for inorganic species sorption studies, such as the one with arsenic [27, 28] and it can be well applied to the purification of organic molecules.

3.1. pH Influence in the Anthocyanin Sorption. The influence of pH on the adsorption of anthocyanins was studied with the purpose of finding out which are the best conditions for the adsorption of these compounds and also to determine the adsorption capacity of the cationic exchange resin loaded with different ions.

Based on the acquired results, a new adsorption mechanism for the main anthocyanin in the resin was proposed. The elution order described in Section 2.3.3 was done according to observations made during the experimentation phase. The metal-anthocyanin complex was stable in basic media, whereas when added to acidic media the bond was broken and the typical anthocyanins coloration (red) was observed. This fact is corroborated with the research reported by Yoshida et al. [22]. The calculation of the distribution coefficient of each one of the metallic ions was made considering the ligand-exchange equilibrium that is carried out in the resin. For the case of the Fe^{3+} we have

$$\text{R-Fe}^{3+} + \text{Cy-3-glc} \longrightarrow \text{R-Fe}^{3+}\text{-Cy-3-glc} + x\text{H}^{+}, \quad (1)$$

where R = polymeric resin and Cy-3-glc = cyanidin-3-glucoside (main anthocyanin).

Therefore the distribution coefficient is calculated in the following way:

$$D = \frac{\text{mmolCy-3-glc}/\text{g}_{\text{resin}}}{\text{mmolCy-3-glc}_{\text{solution}}}. \quad (2)$$

The extraction percentage (%E) is given by the expression:

$$\%E = \frac{D \times \text{g}_{\text{resin}}}{D \times \text{g}_{\text{resin}} + 1} * 100. \quad (3)$$

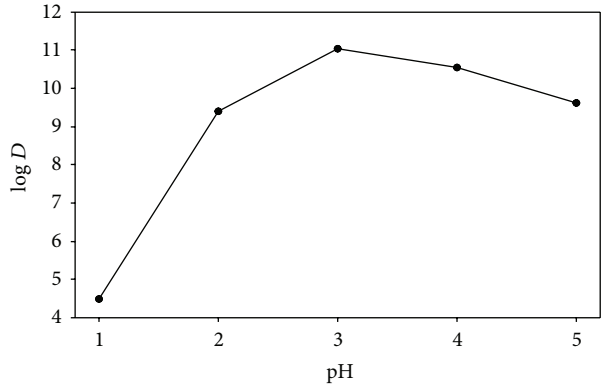


Figure 2: Relationship between distribution coefficient (log D) and pH.

Table 1: Results of sorption anthocyanins processes in a cationic exchange resin loaded with different ions.

Metal ion	log D	%E
Cu(II)	0.52–2.14	7.93–79.81
Ca(II)	0.54–1.74	7.90–57.37
Mg(II)	0.52–1.70	11.12–55.29
Al(III)	0.33–1.76	4.38–56.48
Fe(II)	0.60–1.30	8.96–36.94

On the other hand, the distribution constant is expressed by the next equation:

$$K = \frac{\overline{[\text{Fe-Cy-3-glc}]} \times [\text{H}^+]^x}{\overline{[\text{Fe}^{3+}]} \times [\text{Cy-3-glc}]}, \quad (4)$$

where $\overline{[\text{Fe-Cy-3-glc}]}$, $\overline{[\text{Fe}^{3+}]}$ and [Cy-3-glc] are the concentrations of Cy-3-glc-Fe^{3+} and Fe^{3+} in resin and of Cy-3-glc in solution, respectively.

Defining the distribution coefficient of the anthocyanin in the resin and in the solution we obtained:

$$D = \frac{\overline{[\text{Cy-3-glc}]}}{[\text{Cy-3-glc}]}. \quad (5)$$

Clearing (4) the anthocyanin concentration in the resin ($\overline{[\text{Fe-Cy-3-glc}]} = \overline{[\text{Cy-3-glc}]}$) and replacing it in (5), we get

$$D = \frac{K\left[\text{Fe}^{3+}\right]}{[\text{H}^+]^x}. \quad (6)$$

In logarithmic terms

$$\log D = x\text{pH} + \log\left(K * \left[\text{Fe}^{3+}\right]\right). \quad (7)$$

Equation (7) adopts the form of straight-line equation ($y = Bx + A$). Therefore the slope obtained will be numerically equal to the number of protons that have been liberated or required in the process.

The results found for Cu(II), Ca(II), Mg(II), Al(III), and Fe(II) were shown in Table 1. These metal ions exhibit low values for log D and %E and do not have a linear behavior. Consequently, they were ruled out for further experimentation. These results can be attributed to the fact that the selectivity coefficients of each one of the resins used increase along its corresponding ion valence and size ($M^{4+} > M^{3+} > M^{2+} > M^{+}$) [29]. This shows that the greater the metallic ion concentration is in the resin, the more advantages found in the ligand-exchange mechanism that we propose.

Table 2 shows all of the data that were considered for the D extraction percentage calculations in the method carried out with Fe^{3+}. The initial concentration of total anthocyanins for all pH values was 1.57×10^{-5} mol L^{-1} and was calculated using the spectral data for the Cy-3-glc (ε_{510} = 26900 cm^{-1} mol^{-1} L). The initial concentration of Cy-3-glc was obtained by calculating the percentage of area under the chromatographic peak of this compound in its pure extract form. The percentage obtained was 34.32%. Considering a 100% of area for the sum of all the peaks of the extract and a response factor of 1, the concentration of Cy-3-glc obtained corresponds to 0.3432 ∗ [Initial] and in every case the value found was 5.4×10^{-6} mol L^{-1}. According to the results observed at every range of pH the %E obtained was >99%.

Figure 2 represents the graphic log D versus pH in which it is observed that from pH 3 on, the sorption process is less affected by pH. With this consideration, a correlation between log D versus pH was done. Such results are shown further on.

In Figure 2 it is observed that pH range 1–3 presents a positive slope and pH range 3–5 a negative slope. Thus the correlations log D versus pH were done for these two intervals, finding that for the first of them 3 protons are released ($m = 3.3$, $R^2 = 0.9224$) and in the second range a proton is required so the reaction is carried out ($m = -0.7111$, $R^2 = 0.9702$). This in turn would give origin to the two proposed mechanisms.

Figure 3 shows the ligand-exchange mechanism for the Cy-3-glucoside sorption at the pH interval from 1 to 3. This is the model adjusted to (7) considering a stoichiometry Fe: anthocyanin 1 : 3. In the proposed mechanism it is observed that there is a release of 3 protons which agrees with

Table 2: Results of sorption anthocyanins processes in a cationic exchange resin loaded with Fe^{3+} ions.

pH	$[\text{Cy-3-glc}]_{\text{final}}$, 10^{-6} mol L^{-1}	m_{resin} (g)	log D
1	1.98	0.1530	4.49
2	3.90	0.1725	9.41
3	4.41	0.1758	11.04
4	4.17	0.1694	10.54
5	4.61	0.2161	9.62

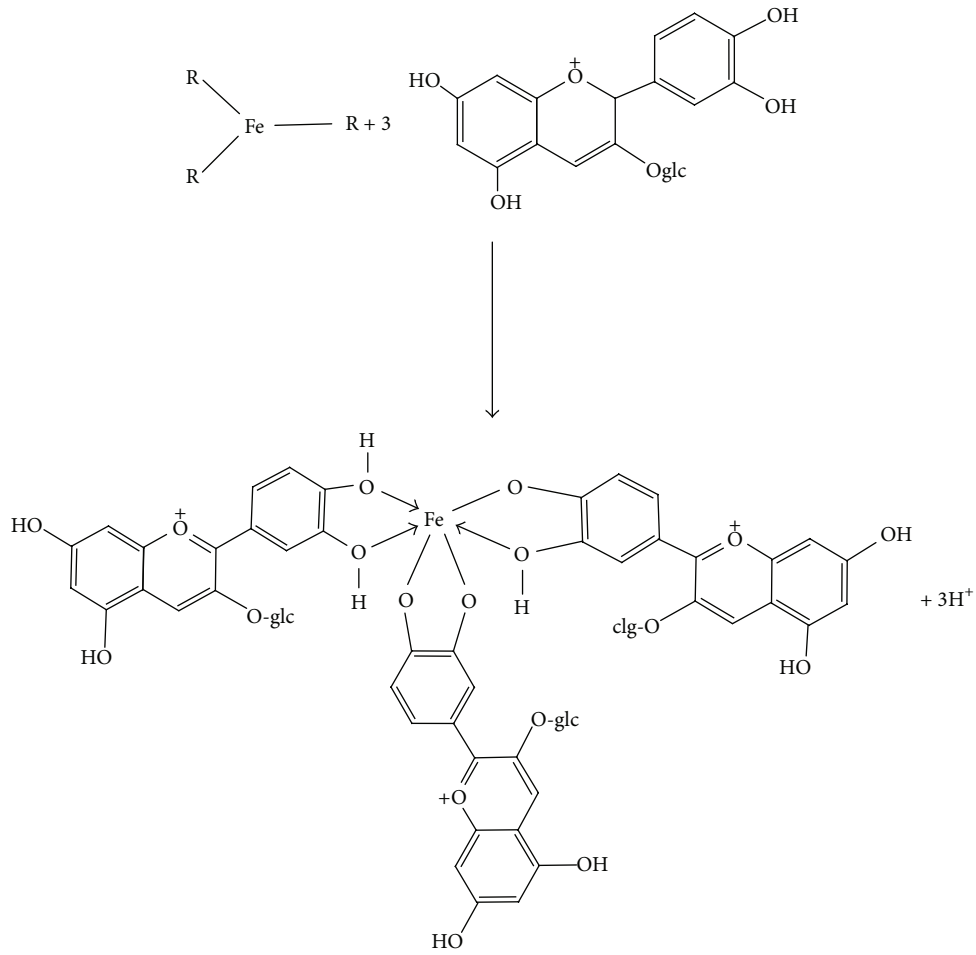


Figure 3: Ligand-exchange mechanism proposed for the Cy-3-glucoside sorption in cationic exchange resin loaded with Fe^{3+}, in the pH range 1–3, where glc = glucoside and R = polymeric resin group (styrene-divinylbenzene)-sulfonate.

the correlation that is obtained from log D versus pH for this interval.

Figure 4 shows the ligand-exchange mechanism proposed for the Cy-3-glucoside sorption in the 3–5 pH interval. The following considerations were done according to the model that better adjusts to (7): (a) that the stoichiometry Fe: anthocyanin was 1 : 1, (b) that Fe(III) was found in the second hydroxycomplex $[Fe(OH)_2{}^+]$, and (c) that the anthocyanin was in its anionic form (considering pK_a's calculated by Moncada et al. [25], pK_a = 3.34).

This experiment was not done at higher pH values because the free anthocyanins in solution are not stable in this region.

3.2. Sorption Experiments at pH 5. According to Figure 2, the greatest value for log D is obtained at pH 3. Nevertheless, it was decided to work at pH 5 to secure the formation of the complex Fe(III)-Anthocyanin as stated by Yoshida et al. [22]. In addition, Fe(III) will be better linked to the resin (more stability) and log D does not change significantly with respect to pH = 3. The respective chromatograms for each one of the elutions were obtained from their HPLC analysis (Figure 5). In the chromatograms it is possible to observe that only one anthocyanin is eluted with HCl 0.1 mol L^{-1} (Fraction D).

These results indicate that only this anthocyanin is capable of forming a complex with Fe^{3+} and elute as an anthocyanin, given that the Fe-anthocyanin complex is dissociated

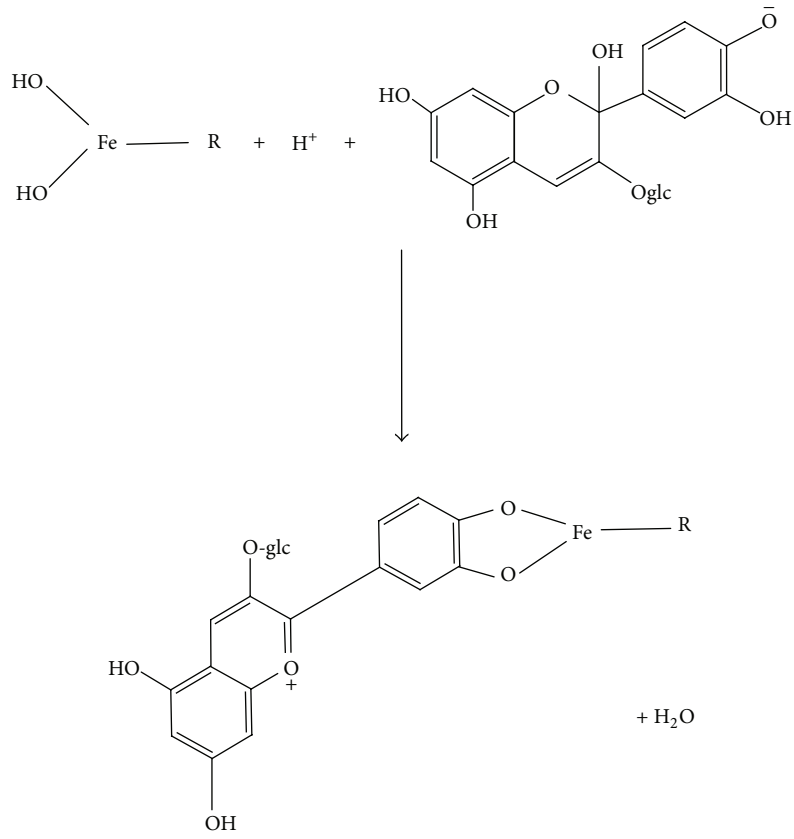


Figure 4: Exchange-ligand mechanism proposed for Cy-3-glucoside sorption in cationic exchange resin loaded with Fe^{3+} from pH 3, where: glc = glucoside and R = polymeric resin group (styrene-divinylbenzene)-sulfonate.

in acidic media and is stable in basic conditions; thus, it is still retained when the elution with NaOH 0.1 mol L^{-1} is carried out.

3.3. Acidity Constants Calculation. The pK_a's calculations of the purified anthocyanin (cyanidin-3-glucoside) were made with the purpose of corroborating the presence of the purified anthocyanin considering pK_a's as a thermodynamic parameter to determine its purity and also to validate the proposed sorption model which adjusts with the species considered in values of pK_a.

Due to previous results obtained by MALDI-ToF MS [24], it was found that Cy-3-glucoside is a component of the main fraction collected in the purification by HPLC from the semipurified anthocyanin extract. And given the complexity of the real system, it was decided to calculate the experimental acidity constants of the anthocyanin purified by the proposed method.

In spectra that were obtained for each solution (Figure 6) a hypochromic effect was observed in the 500–550 nm maximum absorption while the pH value increases. In addition there are changes in absorption bands in the UV region.

For solutions at pH 4, HCl 1 mol L^{-1} was added until the flavylium ion was obtained again (red color). However, it was observed that the color change was not reversible in solutions with a pH 8 or more, thus these data were not considered in acidity constants calculations.

The information processing was carried out in the first place by calculating the number of species that absorb by TRIANG, which is a FORTRAN-based program that determines the most probable number of chemical species that absorb electromagnetic radiation from an array of absorbance at different wavelengths. In general terms, TRIANG is the best strategy to find the number for linearly independent equations. Its algorithm is based on the fact that the matrix of absorptions is reduced to a triangular form and compared with the matrix of absorption errors, which in turn is reduced also to a triangular form. The triangular error matrix is constructed from the value of the error in the transmittance (ΔT) and error propagation equation [30].

The number of species that the program reports is dependent on the value of ΔT, which must be evaluated from the errors involved in the preparation of solutions and the instruments by applying the theory of error propagation. Considering a ΔT from 0.003 to 0.006 ($\Delta T_{\text{spectrophotometer}}$ = 0.003) and obtaining a number of species of four in the interval of pH 1–7 only 3 equilibriums were considered in the refinement of the equilibrium constants by means of the SQUAD program [31].

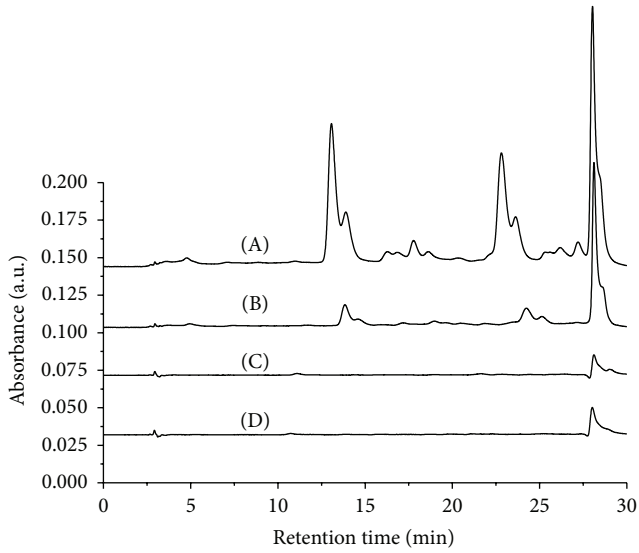


Figure 5: Chromatograms obtained from each one of the fractions of the adsorption process. (A) Extract at pH 5 with acetate buffer. (B) Nonretained fraction. (C) Eluted fraction with NaOH 0.1 mol L^{-1}. (D) Eluted fraction with HCl 0.1 mol L^{-1}.

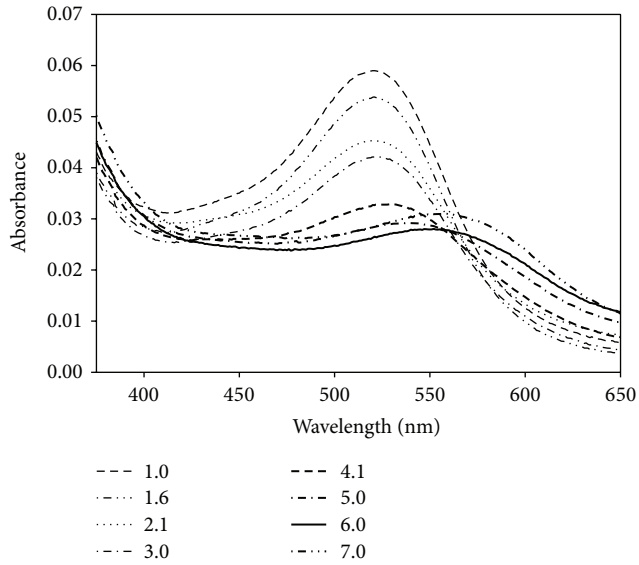


Figure 6: UV-vis absorption spectra for the eluted fraction with HCl 0.1 mol L^{-1} of the sorption experiment at pH 5, in the range 375–650 nm.

SQUAD (stability quotients from absorbance data) is a FORTRAN-based program designed to perform a calculation of the best values for the stability constant from pH spectrophotometric titration data. In the analysis of multicomponent spectra the Beer-Lambert law and the law of an absorbance's additivity are assumed to hold. It calculates the best values for the stability constants and molar absorptivity for the proposed model. Besides, various acid-base equilibria mixed-metal and/or mixed ligand complexes can also be studied, and the program can be used for a treatment of absorbance data from non-aqueous as well as from aqueous media [32].

The program can perform a refining of the resulting data for the stability constants for a general complex $M_pL_qH_j$, where $p, q \gg 0$ and j is positive (for protons), negative (for hydroxide ions), or zero respectively, and by employing a nonlinear least-squares approach. The data fed to the program is the absorbance spectra, a chemical composition (total concentration of M, L and pH), and a chemical model to describe the system. SQUAD computes the values of the overall stability constants which minimize the sum of the squared residuals between the observed and calculated absorbance values, as follows:

$$U = \sum_{k=1}^{q} \sum_{\lambda=1}^{m} \left(A_k^{\text{exper}(\lambda)} - A_k^{\text{calc}(\lambda)} \right)^2. \quad (8)$$

For the constants refinement, a spectral region from 305 to 600 nm was covered, at intervals of 7 nm. The values of

these refined constants were pK_{a1} = 1.59 (0.19), pK_{a2} = 3.94 (0.15), and pK_{a3} = 6.73 (0.11).

Results obtained are very similar to those found for the Cy-3-glucoside standard by Moncada and coworkers [25], whose values are pK_{a1} = 1.85, pK_{a2} = 3.34, and pK_{a3} = 6.60. These results are also different from the constants reported for Malvidin-3-glucoside, whose pK_a's reported are pK_{a1} = 1.76 ± 0.07, pK_{a2} = 5.36 ± 0.04 and pK_{a3} = 8.39 ± 0.07 [33]. In addition, the o-dihydroxy arrangement is not present. The differences between the constants calculated in this work and the ones reported previously are attributed to the lack of control of neither the temperature nor the ionic strength (μ).

These facts apparently confirm that the anthocyanin that forms the complex with Fe^{3+} present in the extract is the Cy-3-glucoside, in addition to covering the complexation requirement that is the o-dihydroxy arrangement in the B-aromatic ring.

4. Conclusions

The proposed Cy-3-glucoside purification method by means of sorption in cationic exchange resin loaded with Fe^{3+} is shown to be quite viable for o-dihydroxy anthocyanins, because of the following.

(1) It decreases procedure costs, since it is possible to be carried out in less time unlike the chromatographic methods proposed previously.

(2) The percentage of anthocyanin extraction reached is above 99.99%.

(3) It is possible to collect the main anthocyanin with high purity and analyze it by other spectroscopic techniques.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

The authors acknowledged SNI (CONACyT) for the stipend received.

References

[1] A. Castañeda-Ovando, M. L. Pacheco-Hernández, M. E. Páez-Hernández et al., "Chemical studies of anthocyanins: a review," *Food Chemistry*, vol. 113, no. 4, pp. 859–871, 2009.

[2] I. Konczak and W. Zhang, "Anthocyanins—more than nature's colours," *Journal of Biomedicine and Biotechnology*, vol. 2004, no. 5, pp. 239–240, 2004.

[3] M. M. Giusti and R. E. Wrolstad, "Unit F1.2.1–13. Anthocyanins. Characterization and measurement of anthocyanins by UV–Visible spectroscopy," in *Current Protocols in Food Analytical Chemistry*, E. R. Wrolstad, Ed., John Wiley & Sons, New York, NY, USA, 2001.

[4] E.-S. M. Abdel-Aal, J. C. Young, and I. Rabalski, "Anthocyanin composition in black, blue, pink, purple, and red cereal grains," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 13, pp. 4696–4704, 2006.

[5] D. Kammerer, J. Gajdos Kljusuric, R. Carle, and A. Schieber, "Recovery of anthocyanins from grape pomace extracts (Vitis vinifera L. cv. Cabernet Mitos) using a polymeric adsorber resin," *European Food Research and Technology*, vol. 220, no. 3-4, pp. 431–437, 2005.

[6] J. Lee, R. W. Durst, and R. E. Wrolstad, "Impact of juice processing on blueberry anthocyanins and polyphenolics: comparison of two pretreatments," *Journal of Food Science*, vol. 67, no. 5, pp. 1660–1667, 2002.

[7] M. P. Kähkönen, A. I. Hopia, and M. Heinonen, "Berry phenolics and their antioxidant activity," *Journal of Agricultural and Food Chemistry*, vol. 49, no. 8, pp. 4076–4082, 2001.

[8] M. R. Coutinho, M. B. Quadri, R. F. P. M. Moreira, and M. G. N. Quadri, "Partial purification of anthocyanins from Brassica oleracea (red cabbage)," *Separation Science and Technology*, vol. 39, no. 16, pp. 3769–3782, 2004.

[9] H. Donner, L. Gao, and G. Mazza, "Separation and characterization of simple and malonylated anthocyanins in red onions, *Allium cepa* L.," *Food Research International*, vol. 30, no. 8, pp. 637–643, 1997.

[10] A. Romani, N. Mulinacci, P. Pinelli, F. F. Vincieri, and A. Cimato, "Polyphenolic content in five tuscany cultivars of Olea europaea L.," *Journal of Agricultural and Food Chemistry*, vol. 47, no. 3, pp. 964–967, 1999.

[11] T. Fossen and Ø. M. Andersen, "Anthocyanins from red onion, Allium cepa, with novel aglycone," *Phytochemistry*, vol. 62, no. 8, pp. 1217–1220, 2003.

[12] M. Schwarz, S. Hillebrand, S. Habben, A. Degenhardt, and P. Winterhalter, "Application of high-speed countercurrent chromatography to the large-scale isolation of anthocyanins," *Biochemical Engineering Journal*, vol. 14, no. 3, pp. 179–189, 2003.

[13] A. M. Vivar-Quintana, C. Santos-Buelga, and J. C. Rivas-Gonzalo, "Anthocyanin-derived pigments and colour of red wines," *Analytica Chimica Acta*, vol. 458, no. 1, pp. 147–155, 2002.

[14] L. Gao and G. Mazza, "Quantitation and distribution of simple and acylated anthocyanins and other phenolics in blueberries," *Journal of Food Science*, vol. 59, no. 5, pp. 1057–1059, 1994.

[15] B. Berente, D. de la Calle García, M. Reichenbacher, and K. Danzer, "Method development for the determination of anthocyanins in red wines by high-performance liquid chromatography and classification of German red wines by means of multivariate statistical methods," *Journal of Chromatography A*, vol. 871, pp. 95–103, 2000.

[16] J. A. Kennedy and A. L. Waterhouse, "Analysis of pigmented high-molecular-mass grape phenolics using ion-pair, normal-phase high-performance liquid chromatography," *Journal of Chromatography A*, vol. 866, no. 1, pp. 25–34, 2000.

[17] E. Mataix and M. D. Luque De Castro, "Determination of anthocyanins in wine based on flow-injection, liquid-solid extraction, continuous evaporation and high-performance liquid chromatography-photometric detection," *Journal of Chromatography A*, vol. 910, no. 2, pp. 255–263, 2001.

[18] N. A. Nyman and J. T. Kumpulainen, "Determination of anthocyanidins in berries and red wine by high-performance liquid chromatography," *Journal of Agricultural and Food Chemistry*, vol. 49, no. 9, pp. 4183–4187, 2001.

[19] C. Alcalde-Eon, M. T. Escribano-Bailón, C. Santos-Buelga, and J. C. Rivas-Gonzalo, "Separation of pyranoanthocyanins from red wine by column chromatography," *Analytica Chimica Acta*, vol. 513, no. 1, pp. 305–318, 2004.

[20] C. T. da Costa, D. Horton, and S. A. Margolis, "Analysis of anthocyanins in foods by liquid chromatography, liquid chromatography-mass spectrometry and capillary electrophoresis," *Journal of Chromatography A*, vol. 881, no. 1-2, pp. 403–410, 2000.

[21] R. Boulton, "The copigmentation of anthocyanins and its role in the color of red wine: a critical review," *American Journal of Enology and Viticulture*, vol. 52, pp. 67–87, 2001.

[22] K. Yoshida, S. Kitahara, D. Ito, and T. Kondo, "Ferric ions involved in the flower color development of the Himalayan blue poppy, Meconopsis grandis," *Phytochemistry*, vol. 67, no. 10, pp. 992–998, 2006.

[23] M. T. Escribano-Bailón, C. Santos-Buelga, and J. C. Rivas-Gonzalo, "Anthocyanins in cereals," *Journal of Chromatography A*, vol. 1054, no. 1-2, pp. 129–141, 2004.

[24] A. Castañeda-Ovando, C. A. Galán-Vidal, L. Pacheco, J. A. Rodriguez, and M. E. Páez-Hernández, "Characterization of main anthocyanins extracted from pericarp blue corn by MALDI-ToF MS," *Food Analytical Methods*, vol. 3, no. 1, pp. 12–16, 2010.

[25] M. C. Moncada, S. Moura, M. J. Melo, A. Roque, C. Lodeiro, and F. Pina, "Complexation of aluminum(III) by anthocyanins and synthetic flavylium salts: a source for blue and purple color," *Inorganica Chimica Acta*, vol. 356, pp. 51–61, 2003.

[26] K. L. Hale, S. P. McGrath, E. Lombi et al., "Molybdenum sequestration in *Brassica* species. A role for anthocyanins?" *Plant Physiology*, vol. 126, no. 4, pp. 1391–1402, 2001.

[27] J. A. Muñoz, A. Gonzalo, and M. Valiente, "Arsenic adsorption by Fe(III)-loaded open-celled cellulose sponge. Thermodynamic and aelectivity aspects," *Environmental Science & Technology*, vol. 36, pp. 3405–3411, 2002.

[28] I. Rau, A. Gonzalo, and M. Valiente, "Arsenic(V) adsorption by immobilized iron mediation: modeling of the adsorption process and influence of interfering anions," *Reactive and Functional Polymers*, vol. 54, no. 1–3, pp. 85–94, 2003.

[29] Dow, "DOWEX Ion Exchange Resins: using ion exchange resin selectivity coefficients," Dow, Midland, Mich, USA, 2008.

[30] F. R. Hartley, C. Burgess, and C. R. M. Alcock, *Solution Equilibria*, Ellis Horwood, New York, NY, USA, 1980.

[31] D. J. Leggett and W. A. E. McBryde, "General computer program for the computation of stability constants from absorbance data," *Analytical Chemistry*, vol. 47, no. 7, pp. 1065–1070, 1975.

[32] D. J. Leggett, *Computational Methods for the Determination of Formation Constants*, Plenum Press, New York, NY, USA, 1985.

[33] R. E. Asenstorfer, P. G. Iland, M. E. Tate, and G. P. Jones, "Charge equilibria and pKa of malvidin-3-glucoside by electrophoresis," *Analytical Biochemistry*, vol. 318, no. 2, pp. 291–299, 2003.

Hindawi Publishing Corporation
Journal of Chemistry
Volume 2014, Article ID 353540, 6 pages
http://dx.doi.org/10.1155/2014/353540

Research Article

Synthesis and Preliminary Properties of Novel Poly(aryl ether)s Containing β-Naphthalene Pendant Group

L. Wang, D. Tao, X. Z. Xiang, and G. M. Zhu

Shenzhen Key Laboratory of Special Functional Materials and College of Materials Science and Engineering, Shenzhen University, Shenzhen 518060, China

Correspondence should be addressed to L. Wang; wl@szu.edu.cn

Received 29 May 2014; Accepted 14 July 2014; Published 28 August 2014

Academic Editor: Waqar Ahmad

Copyright © 2014 L. Wang et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

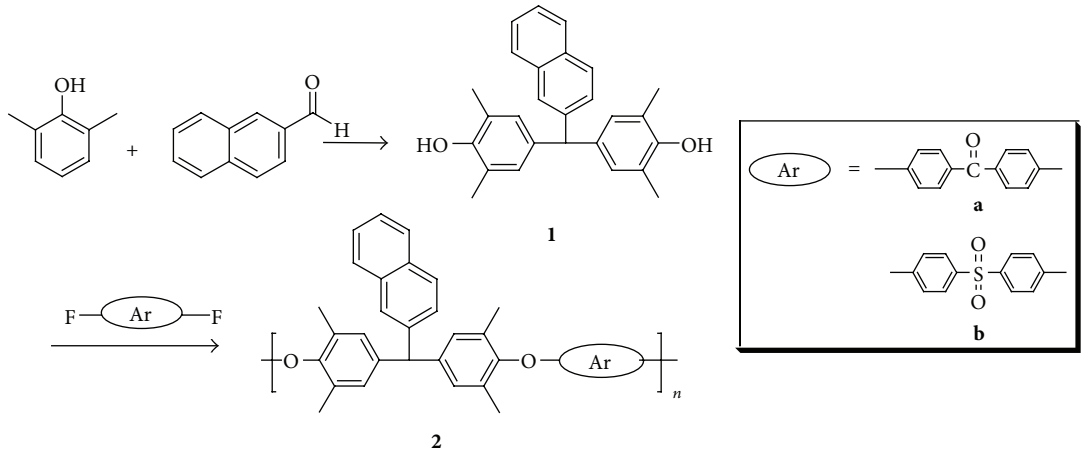
Two novel poly(aryl ether)s containing β-naphthalene pendant group were synthesized and the structures of these polymers were confirmed by ^{1}HNMR spectroscopy. The polymers exhibited good thermal stabilities with high T_g of 256°C and 274°C, respectively. The polymers are soluble in common organic solvents, such as DMAc, DMSO, CH_2Cl_2, and $CHCl_3$, and can be electrospun into microfiber (1–5 μm) with lots of nanopores (<100 nm) from $CHCl_3$ solution. These fibers showed high hydrophobicity, and the contact angle of fibers is above 120°.

1. Introduction

Poly(aryl ether)s [1–10] have extensive applications in aerospace, aviation, electronics, telecommunications, nuclear energy, and other high technology fields because of their excellent properties, such as heat resistance, radiation resistance, impact resistance, and chemical resistance. Poly(aryl ether ketone)s (PAEKs) and poly(aryl ether sulfone)s (PAESs) are two important commercial poly(aryl ether)s. It is difficult for the standard PAEKs to be used as thin films and coating materials because of their semicrystalline property and poor solubility. For PAESs, their solvent resistance is often poor and they could not form oriented film or fiber [11, 12]. Therefore, study on improving the processing properties and solubility of poly(aryl ether)s through structural modification is one of the research hotspots. Structure changes have focused on the introduction of semi flexible groups (such as sulfone, sulfur ether, methylene substituents) to improve the solubility and rigid groups (such as naphthalene [12–14], aromatic heterocyclic, anthracene) to enhance the thermal stabilities, and the incorporation of pendant alkyl or phenyl groups on to the poly(aryl ether) in the backbone of the polymeric chain [15–19]. Our group has synthesized and characterized poly(aryl ether)s with pendant α-naphthyl groups [20]. Recent study indicated that these polymers could be electrospun into microfiber with nanopores [12]. As we know that the properties of compounds substituted with α-naphthalene and β-naphthalene are different, we think that if β-naphthyl groups are introduced into the poly(aryl ether) chain, the properties of the polymers may be enhanced. In order to further study poly(aryl ether)s with pendant naphthyl groups, two novel poly(aryl ether)s were synthesized by using bis(3,5-dimethyl-4-hydroxyphenyl)-β-naphthyl methane and 4,4′-difluorobenzophenone or bis(4-fluorophenyl) sulfone through nucleophilic aromatic substitution. The thermal stabilities and solubility were investigated. In addition, these polymers could be made into fibers and the morphologies of the fibers were also characterized.

2. Experimental Section

2.1. Materials. Bis(3,5-dimethyl-4-hydroxyphenyl)-β-naphthyl methane, 4,4′-difluorobenzophenone, bis(4-fluorophenyl) sulfone, 3-mercaptopropionic acid, and sulfuric acid (60%) were purchased from commercial sources and used as received. Toluene was dried with sodium wire and DMAC was dried with 4 Å molecule sieves prior to use. Anhydrous potassium carbonate was dried at 300°C for 24 h in furnace prior to use.



Scheme 1: Syntheses of monomer **1** and polymers **2**.

2.2. Characterization and Methods. ^{1}H-NMR spectra, reported in ppm, were recorded on a Varian 400 Hz NMR instrument using DMSO-d_6 or chloroform as the solvent and tetramethylsilane (TMS) as the internal standard. The thermal stability of the polymers was evaluated on a Perkin-Elmer TGA/DTA 6300 at a heating rate of 20°C/min under nitrogen. A Perkin-Elmer DSC-7C calorimeter was used to measure the T_g at a heating rate of 20°C/min under nitrogen. The average molecular weights of the polymers were estimated by a Waters 510 HPLC gel permeation chromatography (GPC) system equipped with 5 μm phenol gel columns (linear, 4 × 500 Å) arranged in series using polystyrene as the standard. Microscopic surface morphology of polymer fiber was studied with a JEOL JSM-5610 scanning electron microscope (SEM). Water contact angles of the polymer films on a glass slide were measured with a 59980-35 contact angle analyzer from COLE-PARMER. Melting points were taken on a XT4A melting point apparatus.

*2.3. Syntheses of Monomer **1** and Polymers **2**.* As depicted in Scheme 1, bis (3,5-dimethyl-4-hydroxy phenyl)-2-naphthyl methane (monomer **1**) was synthesized. To a 100 mL three-neck round bottom flask, equipped with a reflux condenser, magnetic stirrer, nitrogeninlet, and a drop funnel, 2-naphthaldehyde (15.6 g, 0.1 mol), 2,6-dimethylphenol (36.6 g, 0.3 mol), 40 mL of toluene were introduced. A portion of 10 mL of 60 wt% H_2SO_4 with 5 drops of 3-mercaptopropionic acid was added dropwise at room temperature for 30 min. The reaction mixture was heated at 50–55°C, stirring for 12 h, until the viscous pink precipitation was produced. The resulting mixture was washed with water 3 times and small amount of toluene 2 times and then recrystallized with toluene 2 times. Finally, we get light-yellow crystal. After filtration, the precipitation was dried at 30°C under vacuum for 24 h. Melting point (T_m) was 166-167°C.

The structure of the monomer was confirmed by ^{1}H NMR spectra (Figure 1). ^{1}HNMR (DMSO-d_6, δppm): 2.102 (s, 12H), 5.399 (s, 1H), 6.688 (s, 4H), 7.295–7.299 (d, 1H), 7.446–7.512 (m, 3H), 7.743–7.845 (m, 3H), 8.097 (s, 2H). Elemental.

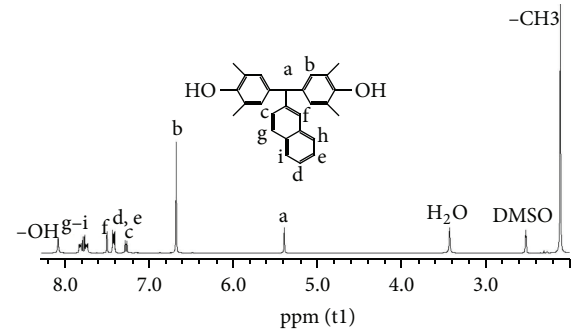


Figure 1: ^{1}H NMR spectrum of monomer **1** in DMSO-d_6.

analysis: calculated for $C_{27}H_{26}O_2$ (382.49): C, 84.78; H, 6.85. Found: C, 84.67; H, 6.80.

The poly(aryl ether)s were prepared as depicted in Scheme 1. To a 25 mL three-neck round bottom flask equipped with a part flow arrangement and condenser under nitrogen. 4,4′-difluorobenzophenone (0.218 g, 1.0 mmol) or bis(4-fluorophenyl) sulfone (0.254 g, 1.0 mmol), bisphenol monomer **1** (0.382 g, 1.0 mmol), anhydrous potassium carbonate (0.208 g, 1.5 mmol), DMAc (2.0 mL), and toluene (5 mL) were carefully introduced. The reaction mixture was first heated at 140–150°C for 4 h to remove the water produced by azeotropic dissolution. Then the temperature was increased to 170–180°C and this temperature was kept for 2-3 h. After it was cooled, the resulting mixture was diluted with 2 mL DMAc and poured into 100 mL methanol containing 2 mL of concentrated HCl slowly to precipitate white nodular solid. The white solid polymer was washed with methanol. After drying, the polymer was dissolved in 50 mL chloroform and filtered through a funnel. The filtrate was concentrated to about 7 mL and then added dropwise into 100 mL methanol to precipitate out the white polymer. After filtration, the precipitation was dried at 120°C under vacuum for 24 h (yield: 92%).

The structure of the polymers was confirmed by ^{1}HNMR spectra (Figure 2). ^{1}HNMR ($CDCl_3$, δppm): polymer **2a**:

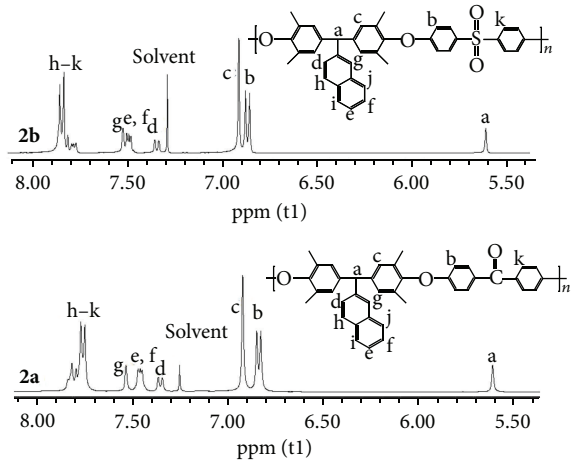


Figure 2: ^{1}H NMR spectrum of polymers **2** in $CDCl_3$.

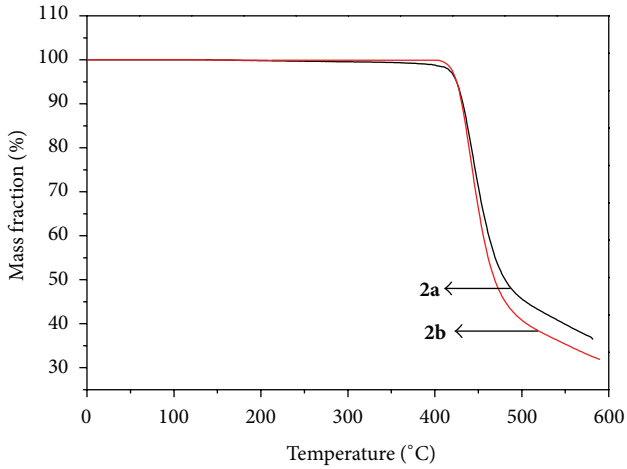


Figure 3: TGA curves of polymers **2**.

2.080 (s, 12H), 5.608 (s, 1H), 6.824–6.845 (m, 4H), 6.919 (s, 4H), 7.341–7.362 (d, 1H), 7.446–7.467 (m, 2H), 7.531 (s, 1H), 7.746–7.768 (m, 5H), 7.814 (m, 2H); polymer **2b**: 2.007 (s, 12H), 5.575 (s, 1H), 6.823–6.845 (m, 4H), 6.880 (s, 4H), 7.300–7.325 (d, 1H), 7.449–7.491 (m, 3H), 7.740–7.783 (m, 5H), 7.902–7.907 (m, 2H).

2.4. Electrospinning Setup. The condition of electrospinning at the room temperature was illustrated as follows: the polymers were dissolved in $CHCl_3$ with a dissolvability of 7.5 wt% (polymer **2a**) and 6 wt% (polymer **2b**), respectively. The solutions were placed in a syringe with metallic needle (inner tip diameter of 1 mm) and a grounded aluminum sheet (thickness, 0.02 mm) was used as the collector. The distance between the spinneret and collector plate was 15 cm. The voltage was 30 kV. The ejection rate of the solution was 5 mL/h. The distance between the tip of the syringe (anode) and the surface of collector (cathode) was 15 cm. The applied direct current voltage was 30 kV. The ejection rate of the solution from the syringe was kept at 5 mL/h and the electrospinning process was carried out in air.

Table 1: Molecular weights and thermal properties of polymers.

Polymer	M_n ($\times 10^4$)	M_w ($\times 10^4$)	T_g (°C)	$T_{5\%}$ (°C)
Polymer **2a**	8.7	16.2	256	418
Polymer **2b**	7.8	13.5	274	424
α-Polymer **a** [20]	9.6	24.4	252	417
α-Polymer **b** [20]	8.9	24.3	273	447

3. Results and Discussion

3.1. Thermal Properties and Hydrophobicity. Thermal properties of the polymers were given in Table 1 and Figure 3. The polymers exhibited excellent thermal properties with 5% mass loss at $T > 400$°C and high T_g of $T > 250$°C. Compared to the results of the polymers containing α-naphthalene groups [20], the T_g slightly raised due to the introduction of β-naphthalene group. With the introduction of bulky β-naphthalene group in the polymer side chain, the chain segment movement steric hindrance increased, so that the T_g of the polymer containing β-naphthalene group increased. The T_g of the polymer **2b** is significantly higher than that of polymer **2a** due to the introduction of the sulfone group. Similar with the polymers containing α-naphthalene groups, the polymers containing β-naphthalene group failed to show the melting points. This indicated they were amorphous in nature. The WAXD spectra showed a diffuse scattering peak from 12° to 25° with no crystallization peak and further confirmed the amorphous nature.

The contact angles both for water and glycerol on polymer films and fiber membranes were measured. The contact angles on both of the fiber membranes were about 120° (part of the contact angles were shown in Figure 4), however, on the films only around 90°. It indicated that the hydrophobicity of poly(aryl ether)s was improved by electrospinning, because the surface free energy of fibers was much higher than that of films.

3.2. Solubility and Morphology. The polymers were soluble in common organic solvents, such as $CHCl_3$, CH_2Cl_2, THF, DMAc, and DMSO, and could be electrospun into microfiber with lots of nanopores. The scanning electron micrographs of polymers **2a** and **2b** fibers were shown in Figures 5 and 6, respectively.

The SEM of the polymer fibers showed that the fibers were all ribbon-shaped, and the average diameters of polymers **2a** and **2b** fibers were about 1 μm and 5 μm, respectively. These poly(aryl ether) fibers are not solid; there are a lot of pores, even the fibers are hollow. Moreover, there were lots of nanopores on the poly(aryl ether) fibers, and many nanopores are communicated with the internal. The nanopores were probably produced by the rapid evaporation of chloroform solvent.

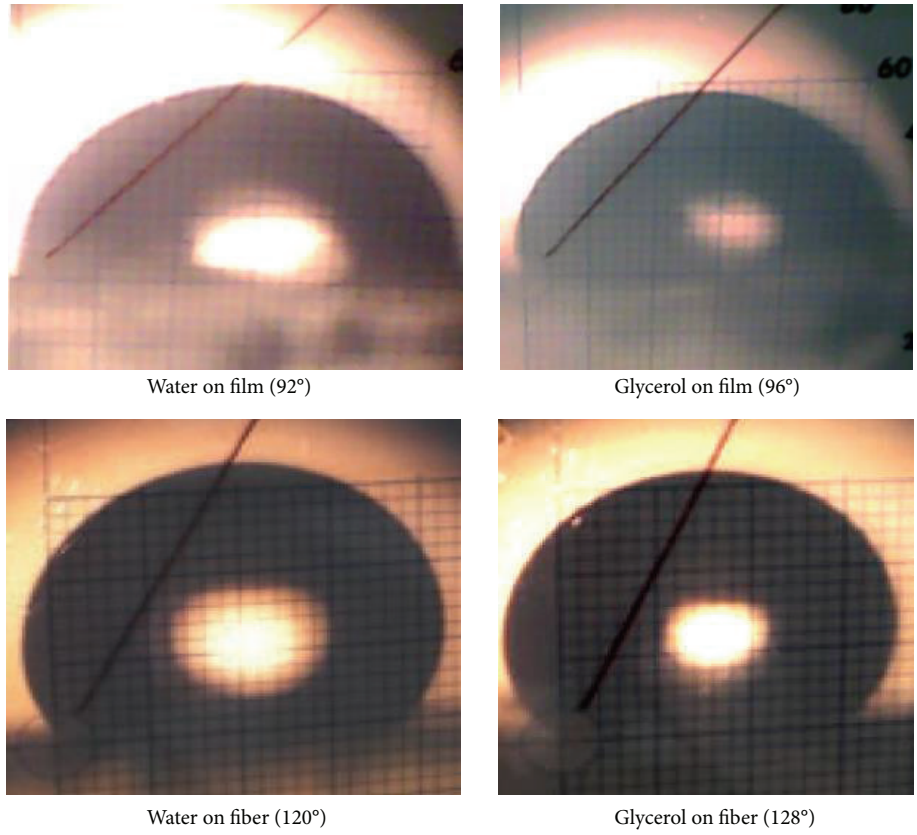


Figure 4: The profiles of a droplet on the polymer **2a** surface.

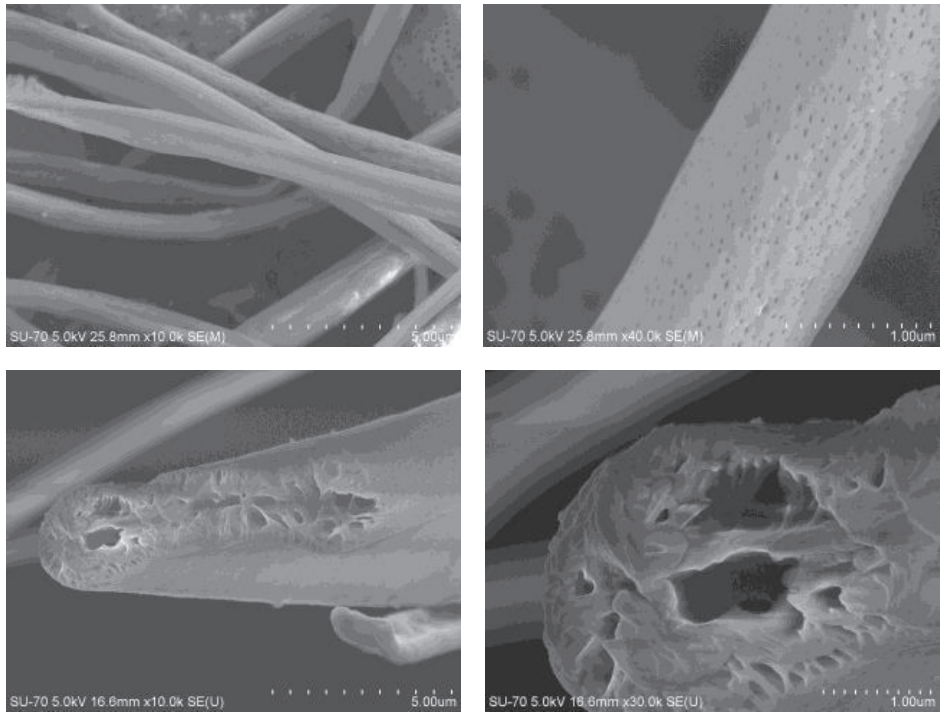


Figure 5: Scanning electron micrographs of polymer **2a** fiber.

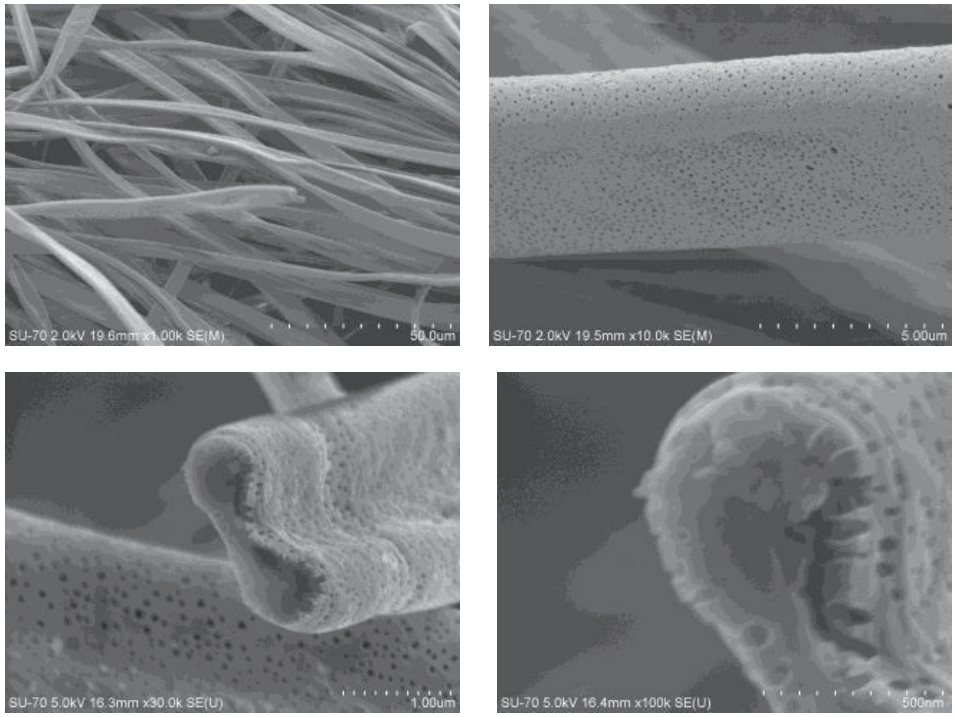


Figure 6: Scanning electron micrographs of polymer **2b** fiber.

4. Conclusion

The novel poly(aryl ether)s containing β-naphthalene pendant group were successfully synthesized. These polymers had excellent thermal stabilities and good solubility. The fibers with many nanopores that were produced by electrospinning exhibited high hydrophobicity. The polymers could be potentially used as high temperature materials, waterproof materials, and transport carriers.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors would like to thank the National Nature Science Foundation of China, China (nos. 51171117, 51003060, and 51101103) and Shenzhen Sci & Tech research Grants (JCYJ20130329105010137, JC2011042100070A, and ZYC201105170225A) for their financial support.

References

[1] M. F. Teasley and B. S. Hsiao, "Synthesis and characterization of poly(oxy-1,3-phenylenecarbonyl-1,4-phenylene) and related polymers," *Macromolecules*, vol. 29, no. 20, pp. 6432–6441, 1996.

[2] P. T. McGrail, "Polyaromatics," *Polymer International*, vol. 41, no. 2, pp. 103–121, 1996.

[3] P. M. Hergenrother, "The use, design, synthesis, and properties of high performance/high temperature polymers: an overview," *High Performance Polymers*, vol. 15, no. 1, pp. 3–45, 2003.

[4] P. A. Staniland, "Poly(ether ketone)s," in *Comprensive Polymer Science*, G. Allen and J. C. Bevington, Eds., vol. 5, pp. 483–497, Pergamon, Oxford, UK, 1989.

[5] K. Tienda, Z. Yu, F. Constandinidis, A. Fortney, W. A. Feld, and E. Fossum, "Poly(arylene ether)s with pendant diphenyl phosphoryl groups: Synthesis, characterization, and thermal properties," *Journal of Polymer Science A Polymer Chemistry*, vol. 49, no. 13, pp. 2908–2915, 2011.

[6] S. Kaiti and E. Fossum, "Effect of reaction conditions on the molecular weight and polydispersity of linear poly(arylene ether phosphine oxide)s prepared from an AB monomer," *Journal of Polymer Science A: Polymer Chemistry*, vol. 44, no. 6, pp. 2099–2106, 2006.

[7] D. P. Bernal, N. Bankey, R. C. Cockayne, and E. Fossum, "Fluoride-terminated hyperbranched poly(arylene ether phosphine oxide)s via nucleophilic aromatic substitution," *Journal of Polymer Science, Part A: Polymer Chemistry*, vol. 40, no. 10, pp. 1456–1467, 2002.

[8] J. H. Pang, K. Z. Shen, D. F. Ren, S. N. Feng, and Z. H. Jiang, "Polyelectrolyte based on tetra-sulfonated poly(arylene ether)s for direct methanol fuel cell," *Journal of Power Sources*, vol. 226, pp. 179–185, 2013.

[9] L. Wang, D. Wang, G. Zhu, and J. Li, "Synthesis and properties of highly branched sulfonated poly(arylene ether)s as proton exchange membranes," *European Polymer Journal*, vol. 47, no. 10, pp. 1985–1993, 2011.

[10] M. Cai, M. Zhu, P. Wang, and C. Song, "Synthesis and properties of novel poly(aryl ether ketone)s containing both diphenyl moiety and amide linkages in the main chains," *Polymer*, vol. 51, no. 6, pp. 1293–1300, 2010.

[11] S. M. Mackinnon, T. P. Bender, and Z. Y. Wang, "Poly(aryl ether)s containing o-terphenyl subunits. II. Random poly(ether sulfone)s," *Journal of Polymer Science A: Polymer Chemistry*, vol. 38, no. 1, pp. 9–17, 2000.

[12] X. Zhao, C. Wang, K. Zhang, Z. Zhou, and M. Zhu, "Soluble poly (aryl ether)s containing naphthalene pendant group: synthesis, characterization and electrospinning," *Colloid and Polymer Science*, vol. 288, no. 8, pp. 907–914, 2010.

[13] L. B. Krentsel, V. V. Makarova, Y. V. Kudryavtsev et al., "Interchain exchange and interdiffusion in blends of poly(ethylene terephthalate) and poly(ethylene naphthalate)," *Polymer Science A*, vol. 51, no. 11-12, pp. 1241–1248, 2009.

[14] Y. Gao, G. P. Robertson, M. D. Guiver, S. D. Mikhailenko, X. Li, and S. Kaliaguine, "Low-swelling proton-conducting copoly(aryl ether nitrile)s containing naphthalene structure with sulfonic acid groups meta to the ether linkage," *Polymer*, vol. 47, no. 3, pp. 808–816, 2006.

[15] Q. Zhang, J. Wang, S. Zhang, and S. Li, "Synthesis and alkaline stability of novel cardo poly(aryl ether sulfone)s with pendent quaternary ammonium aliphatic side chains for anion exchange membranes," *Polymer*, vol. 51, no. 23, pp. 5407–5416, 2010.

[16] M. G. Dhara and S. Banerjee, "Fluorinated high-performance polymers: poly(arylene ether)s and aromatic polyimides containing trifluoromethyl groups," *Progress in Polymer Science*, vol. 35, no. 8, pp. 1022–1077, 2010.

[17] Q. Zhang, F. Gong, S. Zhang, and S. Li, "Novel side-chain-type cardo poly(aryl ether sulfone) bearing pendant sulfoalkyl groups for proton exchange membranes," *Journal of Membrane Science*, vol. 367, no. 1-2, pp. 166–173, 2011.

[18] R. Ponnapati, M. J. Felipe, and R. Advincula, "Electropolymerizable terthiophene-terminated poly(aryl ether) dendrimers with naphthalene and perylene cores," *Macromolecules*, vol. 44, no. 19, pp. 7530–7537, 2011.

[19] X.-F. Li, F. P. V. Paoloni, E. A. Weiber, Z.-H. Jiang, and P. Jannasch, "Fully aromatic ionomers with precisely sequenced sulfonated moieties for enhanced proton conductivity," *Macromolecules*, vol. 45, no. 3, pp. 1447–1459, 2012.

[20] L. Wang and G. M. Zhu, "Synthesis and characterization of novel poly(aryl ether)s containing naphthyl moieties," *Chinese Chemical Letters*, vol. 20, no. 8, pp. 965–968, 2009.