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[enemutlu@hacettepe.edu.tr](mailto:enemutlu@hacettepe.edu.tr) (<mailto:enemutlu@hacettepe.edu.tr>)

### Lorena MEMUSHAJ

Department of Pharmacy, Faculty of Medical Sciences, Aldent University, Tirana, Albania  
[lorena.memushaj@ual.edu.al](mailto:lorena.memushaj@ual.edu.al) (<mailto:lorena.memushaj@ual.edu.al>)

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Department of Forensic Toxicology, Institute of Forensic Sciences, Ankara University, Ankara, Türkiye  
[mgumustas@hotmail.com](mailto:mgumustas@hotmail.com) (<mailto:mgumustas@hotmail.com>)

### Mohd Younis RATHER

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**Débora DUMMER MEIRA**

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debora.dummer.meira@gmail.com (mailto:debora.dummer.meira@gmail.com)

**Derya ÖZSAVCI**

Department of Biochemistry, Faculty of Pharmacy, Marmara University, Istanbul, Türkiye  
derya.ozsavci@marmara.edu.tr (mailto:derya.ozsavci@marmara.edu.tr)

**Emine TERZİ**

Department of Medical Biology, Faculty of Medicine, Ankara Yıldırım Beyazıt University, Ankara, Türkiye  
emineterzi1990@hotmail.com (mailto:emineterzi1990@hotmail.com)

**Gülberk UÇAR**

Department of Biochemistry, Faculty of Pharmacy, Hacettepe University, Ankara, Türkiye  
gulberk@hacettepe.edu.tr (mailto:gulberk@hacettepe.edu.tr)

**Haidar A ABDULAMIR**

College of Pharmacy, Al-Maaql University, Basra, Iraq  
h\_al\_attar@yahoo.com (mailto:h\_al\_attar@yahoo.com)

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Department of Biochemistry, Faculty of Pharmacy, Hacettepe University, Ankara, Türkiye  
senaozbay@hacettepe.edu.tr (mailto:senaozbay@hacettepe.edu.tr)

**Işıl YILDIRIM**

Pharmacy Services Program, Beykent University, Istanbul, Türkiye  
assistant.professor.isil.yildirim@gmail.com (mailto:assistant.professor.isil.yildirim@gmail.com)

**Lokman AYAZ**

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lokmanayaz@yahoo.com (mailto:lokmanayaz@yahoo.com)

**Lynda BOUREBABA**

Department of Experimental Biology, Faculty of Biology and Animal Science, Wrocław University of Environmental and Life Sciences, Wrocław, Poland  
lynda.bourebaba@upwr.edu.pl (mailto:lynda.bourebaba@upwr.edu.pl)

**Nadia M. HAMDY**

Department of Biochemistry, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt  
nadia\_hamdy@pharma.asu.edu.eg (mailto:nadia\_hamdy@pharma.asu.edu.eg)

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Nutrition and Food Sciences Department, National Research Centre, Cairo, Egypt  
s\_y\_alokbi@hotmail.com (mailto:s\_y\_alokbi@hotmail.com)

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Laboratory of Applied Biochemistry, Faculty of Natural and Life Sciences, University Ferhat Abbas, Setif, Algeria  
houchi.selma@univ-setif.dz (mailto:houchi.selma@univ-setif.dz)

## *Biotechnology*

### **Ali Demir SEZER**

Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Marmara University, Istanbul, Türkiye  
adsezer@marmara.edu.tr (mailto:adsezer@marmara.edu.tr)

### **Ammad Ahmad FAROOQI**

Department of Molecular Oncology, Institute of Biomedical and Genetic Engineering (IBGE), Islamabad, Pakistan  
farooqiammadahmad@gmail.com (mailto:farooqiammadahmad@gmail.com)

### **Ceyda EKENTOK ATICI**

Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Marmara University, Istanbul, Türkiye  
ceyda.ekentok@marmara.edu.tr (mailto:ceyda.ekentok@marmara.edu.tr)

### **Fahima DILNAWAZ**

School of Engineering and Technology, Centurion University of Technology and Management, Odisha, INDIA  
fahimadilnawaz@gmail.com (mailto:fahimadilnawaz@gmail.com)

### **Murat DOĞAN**

Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Cumhuriyet University, Sivas, Türkiye  
mdogan@cumhuriyet.edu.tr (mailto:mdogan@cumhuriyet.edu.tr)

### **Uğur KARAGÖZ**

Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Trakya University, Edirne, Türkiye  
ugurkaragoz@trakya.edu.tr (mailto:ugurkaragoz@trakya.edu.tr)

## *Clinical and Social Pharmacy & Pharmacoeconomy & Pharmacy Education*

### **Abdikarim Mohammed ABDI**

Department of Clinical Pharmacy, Faculty of Pharmacy, Yeditepe University, Istanbul, Türkiye  
abdikarim.abdi@yeditepe.edu.tr (mailto:abdikarim.abdi@yeditepe.edu.tr)

### **Ahmed Hamza AL-SHAMMARI**

Department of Pharmacy, Kut University College, Alkut, Wasit, Iraq  
Ahmedhamzamezaal@gmail.com (mailto:Ahmedhamzamezaal@gmail.com)

### **Betül OKUYAN**

Department of Clinical Pharmacy, Faculty of Pharmacy, Marmara University, Istanbul, Türkiye  
betul.okuyan@marmara.edu.tr (mailto:betul.okuyan@marmara.edu.tr)

### **Emre KARA**

Department of Clinical Pharmacy, Faculty of Pharmacy, Hacettepe University, Ankara, Türkiye  
emrekara@hacettepe.edu.tr (mailto:emrekara@hacettepe.edu.tr)

### **Ermelinda DURMISHI**

Director, Higher Education and Scientific Research Policies Department, Ministry of Education and Sports, Tirana, Albania  
eridurmishi@yahoo.com (mailto:eridurmishi@yahoo.com)

### **Maja ORTNER HADŽIABDIĆ**

Centre for Applied Pharmacy, Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Croatia  
mortner@pharma.hr (mailto:mortner@pharma.hr)

### **Mesut SANCAR**

Department of Clinical Pharmacy, Faculty of Pharmacy, Marmara University, Istanbul, Türkiye  
mesut.sancar@marmara.edu.tr (mailto:mesut.sancar@marmara.edu.tr)



Faculty of Pharmacy, University of Medicine, Tirana, Albania  
mirela.miraci@umed.edu.al (mailto:mirela.miraci@umed.edu.al)

**Nasir IDKAIDEK**

Department of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmacy and Medical Sciences, Petra University, Amman, Jordan  
nidkaidek@uop.edu.jo (mailto:nidkaidek@uop.edu.jo)

**Tarik CATIĆ**

Department of Pharmacy, Sarajevo School of Science and Technology, Sarajevo, Bosnia and Herzegovina  
tarik.catic@ssst.edu.ba (mailto:tarik.catic@ssst.edu.ba)

*General Chemistry*

**Sinem GÖKTÜRK**

Department of Basic Pharmaceutical Sciences, Faculty of Pharmacy, Marmara University, İstanbul, Türkiye  
sgokturk@marmara.edu.tr (mailto:sgokturk@marmara.edu.tr)

*In Silico Studies*

**Berna DOĞAN**

Department of Chemistry, Faculty of Science and Letters, Istanbul Technical University, Istanbul, Türkiye  
bernadogan@itu.edu.tr (mailto:bernadogan@itu.edu.tr)

**Gizem TATAR YILMAZ**

Department of Biostatistics and Medical Informatics, Faculty of Medicine, Karadeniz Technical University, Trabzon, Türkiye  
gizemtatar@gmail.com (mailto:gizemtatar@gmail.com)

**Onur SERÇİNOĞLU**

Department of Bioengineering, Faculty of Engineering, Gebze Technical University, Kocaeli, Türkiye  
osercinoglu@gtu.edu.tr (mailto:osercinoglu@gtu.edu.tr)

**Mehmet ÖZBİL**

Department of Bioengineering, Faculty of Engineering, Gebze Technical University, Kocaeli, Türkiye  
mozbil@gtu.edu.tr (mailto:mozbil@gtu.edu.tr)

*Medicinal Chemistry*

**Bahadır BÜLBÜL**

Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Düzce University, Düzce, Türkiye  
bahadir.bulbul@yahoo.com.tr (mailto:bahadir.bulbul@yahoo.com.tr)

**Efe Doğukan DİNCEL**

Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Istanbul University, Istanbul, Türkiye  
efe.dincel@istanbul.edu.tr (mailto:efe.dincel@istanbul.edu.tr)

**Entela HALOCI**

Faculty of Pharmacy, University of Medicine, Tirana, Albania  
entela.haloci@umed.edu.al (mailto:entela.haloci@umed.edu.al)

**Göknil Pelin COŞKUN**

Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Acıbadem University, Istanbul, Türkiye  
pelin.coskun@acibadem.edu.tr (mailto:pelin.coskun@acibadem.edu.tr)

Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Karadeniz Technical University, Trabzon, Türkiye  
esellitepe@ktu.edu.tr (mailto:esellitepe@ktu.edu.tr)

**Kerem BURAN**

Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Health Sciences, Istanbul, Türkiye  
Kerem.buran@sbu.edu.tr (mailto:Kerem.buran@sbu.edu.tr)

**Simone CARRADORI**

Department of Pharmacy, "G. d'Annunzio" University of Chieti-Pescara, Chieti, Italy  
simone.carradori@unich.it (mailto:simone.carradori@unich.it)

**Somaieh SOLTANI**

Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran  
soltanis@tbzmed.ac.ir (mailto:soltanis@tbzmed.ac.ir)

*Microbiology & Immunology*

**Shahram KHADEM VATAN**

Department of Medical Parasitology and Mycology, Urmia University of Medical Sciences, Urmia, Iran  
Cellular and Molecular Research Center, Cellular and Molecular Medicine Research Institute, Urmia University of  
Medical Sciences, Urmia, Iran  
Khademvatan@yahoo.com (mailto:Khademvatan@yahoo.com)

**Erkan RAYAMAN**

Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Marmara University, Istanbul, Türkiye  
erayaman@marmara.edu.tr (mailto:erayaman@marmara.edu.tr)

**Gülgün TINAZ**

Department of Basic Pharmaceutical Sciences, Faculty of Pharmacy, Marmara University, Istanbul, Türkiye  
gulgun.tinaz@marmara.edu.tr (mailto:gulgun.tinaz@marmara.edu.tr)

**Zahraa AMER HASHIM**

Department of Microbiology and Immunology, College of Pharmacy, Mosul University, Mosul, Iraq  
hashimz@uomosul.edu.iq (mailto:hashimz@uomosul.edu.iq)

*Pharmaceutical Botany & Pharmacognosy & Chemistry of Natural Products*

**Ahmet EMİR**

Department of Pharmacognosy, Faculty of Pharmacy, Ege University, Izmir, Türkiye  
ahmet.emir@ege.edu.tr (mailto:ahmet.emir@ege.edu.tr)

**Annalisa CHIAVAROLI**

Department of Pharmacology, Faculty of Pharmacy, G. d'Annunzio University of Chieti-Pescara, Chieti, Italy  
annalisa.chiavaroli@unich.it (mailto:annalisa.chiavaroli@unich.it)

**Antoaneta TREDAFILOVA**

Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria  
antoaneta.trendafilova@orgchm.bas.bg (mailto:antoaneta.trendafilova@orgchm.bas.bg)

**Ayşe Esra KARADAĞ**

Department of Pharmacognosy, Faculty of Pharmacy, Istanbul Medipol University, Istanbul, Türkiye  
ayseesraguler@gmail.com (mailto:ayseesraguler@gmail.com)

**Ceren EMİR**

Department of Pharmacognosy, Faculty of Pharmacy, Ege University, Izmir, Türkiye  
ceren.acir@ege.edu.tr (mailto:ceren.acir@ege.edu.tr)

Department of Pharmacology, Faculty of Pharmacy, G. d'Annunzio University of Chieti-Pescara, Chieti, Italy  
claudio.ferrante@unich.it (mailto:claudio.ferrante@unich.it)

**İlker DEMİRBOLAT**

Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Acıbadem University, Istanbul, Türkiye  
ilker.demirbolat@acibadem.edu.tr (mailto:ilker.demirbolat@acibadem.edu.tr)

**İ. İrem TATLI ÇANKAYA**

Department of Pharmaceutical Botany, Faculty of Pharmacy, Hacettepe University, Ankara, Türkiye  
iremcanakaya@gmail.com (mailto:iremcanakaya@gmail.com)

**Laleh KHODAIE**

Department of Pharmacognosy, Faculty of Traditional Medicine, Tabriz University of Medical Sciences, Tabriz, Iran  
khodaiei@gmail.com (mailto:khodaiei@gmail.com)

**Lejla KLEPO**

Department of Chemistry, Faculty of Science, University of Sarajevo, Sarajevo, Bosnia and Herzegovina  
klepolejla@gmail.com (mailto:klepolejla@gmail.com)

**Mirjana MARČETIĆ**

Department of Pharmacognosy, Faculty of Pharmacy, University of Belgrade, Belgrade, Serbia  
mirjana.marctic@pharmacy.bg.ac.rs (mailto:mirjana.marctic@pharmacy.bg.ac.rs)

**Nurettin YAYLI**

Department of Pharmacognosy, Faculty of Pharmacy, Karadeniz Technical University, Trabzon, Türkiye  
yayli@ktu.edu.tr (mailto:yayli@ktu.edu.tr)

**Patrícia RIJO**

Research Center for Biosciences & Health Technologies, Lusofona University, Lisbon, Portugal  
p1609@ulusofona.pt (mailto:p1609@ulusofona.pt)

*Pharmacognosy*

**Sneha AGRAWAL**

Department of Pharmacognosy, Bharati Vidyapeeth's College of Pharmacy, Navi Mumbai, Maharashtra, India  
sneha.agrawal@bvcop.in (mailto:sneha.agrawal@bvcop.in)

**Turgut TAŞKIN**

Department of Pharmacognosy, Faculty of Pharmacy, Marmara University, Istanbul, Türkiye  
turguttaskin@marmara.edu.tr (mailto:turguttaskin@marmara.edu.tr)

**Viktorija MAKSIMOVA**

Department of Applied Sciences, Faculty of Medical Sciences, Goce Delcev University, Shtip, Republic of N. Macedonia  
viktorija.maksimova@ugd.edu.mk (mailto:viktorija.maksimova@ugd.edu.mk)

**Vildan ÇELİKSOY**

School of Optometry and Vision Sciences, Cardiff University, Cardiff, UK  
celiksoyv92@gmail.com (mailto:celiksoyv92@gmail.com)

**Vilma TOSKA PAPAJANI**

Department of Pharmacy, University of Medicine, Tirana, Albania  
toskavilma@gmail.com (mailto:toskavilma@gmail.com)

**Zoran ZEKOVIĆ**

Faculty of Technology, University of Novi Sad, Novi Sad, Serbia  
zzekovic@tf.uns.ac.rs (mailto:zzekovic@tf.uns.ac.rs)

*Pharmaceutics*

Department of Pharmaceutical Sciences and Technology, Birla Institute of Technology, Mesra, India  
monika.nbri@gmail.com (mailto:monika.nbri@gmail.com)

**Afife Büşra UĞUR KAPLAN**

Department of Pharmaceutical Technology, Faculty of Pharmacy, Atatürk University, Erzurum, Türkiye  
afife.busra.ugur@gmail.com (mailto:afife.busra.ugur@gmail.com)

**Rajanikant PATEL**

Granules Pharmaceuticals Inc., Chantilly, VA - 20151, USA  
rajnipharmacy@gmail.com (mailto:rajnipharmacy@gmail.com)

**Burcu ÜNER**

Pharmaceutical and Administrative Sciences, The University of Health Science and Pharmacy in St. Louis, USA  
uner.burcu@yahoo.com (mailto:uner.burcu@yahoo.com)

**Dhanashree P. SANAP**

Department of Pharmaceutics, Bharati Vidyapeeth's College of Pharmacy, Navi Mumbai, India  
dhanashree.sanap@bvcop.in (mailto:dhanashree.sanap@bvcop.in)

**Dinesh KUMAR**

Department of Pharmaceutical Engineering & Technology, Indian Institute of Technology (BHU), Varanasi, India  
dinesh.phe@itbhu.ac.in (mailto:dinesh.phe@itbhu.ac.in)

**Ebru ALTUNTAŞ**

Department of Pharmaceutical Technology, Faculty of Pharmacy, Istanbul University, Istanbul, Türkiye  
ebru.altuntas@istanbul.edu.tr (mailto:ebru.altuntas@istanbul.edu.tr)

**Ela HOTI**

Faculty of Pharmacy, University of Medicine, Tirana, Albania  
ela.hoti@umed.edu.al (mailto:ela.hoti@umed.edu.al)

**Emrah ÖZAKAR**

Department of Pharmaceutical Technology, Faculty of Pharmacy, Atatürk University, Erzurum, Türkiye  
emrahozakar@atauni.edu.tr (mailto:emrahozakar@atauni.edu.tr)

**Enkelejda GOCI**

Pharmacotherapeutic Research Center, Aldent University, Tirana, Albania  
enkelejda.goci@ual.edu.al (mailto:enkelejda.goci@ual.edu.al)

**Kleva SHPATI**

Department of Pharmacy, Albanian University, Tirana, Albania  
k.shpati@albanianuniversity.edu.al (mailto:k.shpati@albanianuniversity.edu.al)

**Sakine TUNCAY TANRIVERDİ**

Department of Pharmaceutical Technology, Faculty of Pharmacy, Ege University, İzmir, Türkiye  
sakine.tuncay@ege.edu.tr (mailto:sakine.tuncay@ege.edu.tr)

**Gülşah GEDİK**

Department of Pharmaceutical Technology, Faculty of Pharmacy, Trakya University, Edirne, Türkiye  
gulsahgedik@trakya.edu.tr (mailto:gulsahgedik@trakya.edu.tr)

**Ongun Mehmet SAKA**

Department of Pharmaceutical Technology and Biotechnology, Faculty of Pharmacy, Ankara University, Ankara, Türkiye  
omsaka@gmail.com (mailto:omsaka@gmail.com)

**Oya KERİMOĞLU**

Department of Pharmaceutical Technology, Faculty of Pharmacy, Marmara University, Istanbul, Türkiye  
osipahigil@marmara.edu.tr (mailto:osipahigil@marmara.edu.tr)

Pharmaceutical and Administrative Sciences, The University of Health Science and Pharmacy in St. Louis, USA  
dwivedipank@gmail.com (mailto:dwivedipank@gmail.com)

**Rezarta SHKRELI**

Department of Pharmacy, Faculty of Medical Sciences, Aldent University, Tirana, Albania  
rezarta.shkreli@ual.edu.al (mailto:rezarta.shkreli@ual.edu.al)

**Renuka KHATIK**

Washington University in St. Louis, USA  
renukadops@gmail.com (mailto:renukadops@gmail.com)

**Rukiye SEVİNÇ ÖZAKAR**

Department of Pharmaceutical Technology, Faculty of Pharmacy, Atatürk University, Erzurum, Türkiye  
rukiyeso@atauni.edu.tr (mailto:rukiyeso@atauni.edu.tr)

**Saeideh SOLTANI**

Novel Drug Research Center, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran  
soltanisa@pharm.mui.ac.ir (mailto:soltanisa@pharm.mui.ac.ir)

*Pharmacology & Toxicology*

**Ana V. PEJČIĆ**

Department of Pharmacology and Toxicology, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia  
anapejic201502@yahoo.com (mailto:anapejic201502@yahoo.com)

**Ayfer BECEREN**

Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Marmara University, Istanbul, Türkiye  
ayfer.tozan@marmara.edu.tr (mailto:ayfer.tozan@marmara.edu.tr)

**Ayşenur GÜNAYDIN AKYILDIZ**

Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Bezmialem Vakıf University, Istanbul, Türkiye  
gunaydinaysenur@gmail.com (mailto:gunaydinaysenur@gmail.com)

**Ayça TOPRAK SEMİZ**

Vocational School of Health Services, Giresun University, Giresun, Türkiye  
ayca.toprak@giresun.edu.tr (mailto:ayca.toprak@giresun.edu.tr)

**Büşra ERTAŞ**

Department of Pharmacology, Faculty of Pharmacy, Marmara University, Istanbul, Türkiye  
busra.ertas@marmara.edu.tr (mailto:busra.ertas@marmara.edu.tr)

**Vasudevan MANI**

Department of Pharmacology and Toxicology, College of Pharmacy, Qassim University, Al Qassim, Kingdom of Saudi Arabia  
v.samy@qu.edu.sa (mailto:v.samy@qu.edu.sa)

**Fatiha MISSOUN**

Laboratory of Pharmacognosy and Api-Phytotherapy, University of Mostaganem, Mostaganem, Algeria  
fatiha.missoun@univ-mosta.dz (mailto:fatiha.missoun@univ-mosta.dz)

**Klodiola DHAMO**

Faculty of Technical Medical Sciences, Aldent University, Tirana, Albania  
klodiola.dhamo@ual.edu.al (mailto:klodiola.dhamo@ual.edu.al)

School of Medical and Life Sciences, Sunway University, Sunway City, Malaysia  
longchiauming@gmail.com (mailto:longchiauming@gmail.com)

**Merve KABASAKAL**

Department of Medical Pharmacology, Faculty of Medicine, University of Health Sciences, Istanbul, Türkiye  
merve.kabasakal@sbu.edu.tr (mailto:merve.kabasakal@sbu.edu.tr)

**Miloš N. MILOSAVLJEVIĆ**

Department of Pharmacology and Toxicology, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia  
milosavljevicmilos91@gmail.com (mailto:milosavljevicmilos91@gmail.com)

**Mohammed Jabbar MANNA**

Department of Pharmacology, College of Dentistry, Al-Mustansiriya University, Baghdad, Iraq  
mohammedalmanna@uomustansiriyah.edu.iq (mailto:mohammedalmanna@uomustansiriyah.edu.iq)

**Nurdan TEKİN**

Department of Medical Pharmacology, Faculty of Medicine, University of Health Sciences, Istanbul, Türkiye  
nurdan.tekin@sbu.edu.tr (mailto:nurdan.tekin@sbu.edu.tr)

**Rümeysa KELEŞ KAYA**

Department of Medical Pharmacology, Faculty of Medicine, Sakarya University, Sakarya, Türkiye  
rumeysakeles@sakarya.edu.tr (mailto:rumeysakeles@sakarya.edu.tr)

**Sana REHMAN**

Department of Pharmacology, HIMSR & HAHC Hospital, Jamia Hamdard, New Delhi, INDIA  
drsanarehman2012@gmail.com (mailto:drsanarehman2012@gmail.com)

**Ünzile YAMAN**

Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Katip Çelebi University, İzmir, Türkiye  
unzileyaman@gmail.com (mailto:unzileyaman@gmail.com)

**Zarife Nigar ÖZDEMİR KUMRAL**

Department of Physiology, Faculty of Medicine, Marmara University, Istanbul, Türkiye  
znozdemir@marmara.edu.tr (mailto:znozdemir@marmara.edu.tr)

**Zeina ALTHANOON**

Department of Pharmacology and Toxicology, College of Pharmacy, Mosul University, Mosul, Iraq  
dr.zeina@uomosul.edu.iq (mailto:dr.zeina@uomosul.edu.iq)

*Copy Editor*

**Ayşe Nur HAZAR YAVUZ**

Department of Pharmacology, Faculty of Pharmacy, Marmara University, Istanbul, Türkiye  
ayse.hazar@marmara.edu.tr (mailto:ayse.hazar@marmara.edu.tr)

**Büşra ERGEN**

Department of Clinical Pharmacy, Faculty of Pharmacy, Marmara University, Istanbul, Türkiye  
busra.ergen@marmara.edu.tr (mailto:busra.ergen@marmara.edu.tr)

**Elif Beyzanur POLAT**

Department of Pharmacology, Faculty of Pharmacy, Marmara University, Istanbul, Türkiye  
elif.beyzanur@marmara.edu.tr (mailto:elif.beyzanur@marmara.edu.tr)

**Fatih Taha ÇİFTÇİ**

Department of Clinical Pharmacy, Faculty of Pharmacy, Marmara University, Istanbul, Türkiye  
fatih.ciftci@marmara.edu.tr (mailto:fatih.ciftci@marmara.edu.tr)

**Müzeyyen AKSOY**

Department of Clinical Pharmacy, Faculty of Pharmacy, Marmara University, Istanbul, Türkiye  
muzeyyen.aksoy@marmara.edu.tr (mailto:muzeyyen.aksoy@marmara.edu.tr)

Department of Clinical Pharmacy, Faculty of Pharmacy, Marmara University, Istanbul, Türkiye  
oozkanli@marmara.edu.tr (mailto:oozkanli@marmara.edu.tr)

**Sinan SERMET**

Istinye University Faculty of Medicine, Department of Clinical Sciences and Department of Pharmacology and Clinical  
Pharmacology, Istanbul, Türkiye  
sinan.sermet@istinye.edu.tr (mailto:sinan.sermet@istinye.edu.tr)

**Şeyma GÖZELİZMİR**

Department of Clinical Pharmacy, Faculty of Pharmacy, Marmara University, Istanbul, Türkiye  
seyma.gozelizmir@marmara.edu.tr (mailto:seyma.gozelizmir@marmara.edu.tr)

**Yeliz ŞAHİN**

Department of Clinical Pharmacy, Faculty of Pharmacy, Marmara University, Istanbul, Türkiye  
yeliz.sahin@marmara.edu.tr (mailto:yeliz.sahin@marmara.edu.tr)

*Language Editor*

**Khadija ALJESRI**

Department of Pharmacology, Institute of Health Sciences, Marmara University, Istanbul, Türkiye

*Biostatistics Editor*

**Gülnaz NURAL BEKİROĞLU**

Department of Biostatistics, Faculty of Medicine, Marmara University, Istanbul, Türkiye  
nural@marmara.edu.tr (mailto:nural@marmara.edu.tr)

Marmara University

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# BBD-driven optimization of an RP-HPLC method for simultaneous analysis of two major isoflavone aglycones in Tofu

Florentinus Dika Octa RISWANTO <sup>1</sup>, Dina Christin Ayuning PUTRI <sup>2</sup>, Michael Raharja GANI <sup>1\*</sup>

<sup>1</sup> Division of Pharmaceutical Analysis and Medicinal Chemistry, Faculty of Pharmacy, Universitas Sanata Dharma, Yogyakarta, Indonesia

<sup>2</sup> Division of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmacy, Universitas Sanata Dharma, Yogyakarta, Indonesia

\* Corresponding Author. E-mail: mr\_gani@usd.ac.id (M.R.G.); Tel. +62-274-883037.

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**ABSTRACT:** Tofu, one of soy food products widely consumed in Indonesia, has been reported as a source of natural protein. It is important to evaluate the contents of genistein and daidzein to provide information according to the nutrient information on soy food products. A reversed-phase high performance liquid chromatography (RP-HPLC) was developed in this study in order to analyze genistein and daidzein content in tofu samples simultaneously. Employing a Box-Behnken design (BBD) for designing the experiment, the response surface methodology (RSM) was then applied to optimize chromatographic conditions including methanol composition, flowrate, and column temperature. Separation response such as retention time, resolution, and tailing factor were evaluated to build an optimization model followed by analyzing the desirability. It was found that the model predicted a composite desirability of 0.9778 can be obtained by applying the methanol composition of 60%, flowrate of 0.80 mL.min<sup>-1</sup>, and column temperature of 50°C. The optimized HPLC conditions met the acceptance criteria for retention time and area deviations, resolution, tailing factor, and theoretical plates number.

**KEYWORDS:** daidzein; genistein; experimental design; tofu.

## 1. INTRODUCTION

Soy food products were widely consumed as functional food mostly in Asian countries. The research interest in soy food products increased since several health benefits have been reported such as agent of breast cancer, prostate cancer, gastrointestinal cancer, endometrial and ovarian cancer, antidiabetics, reproductive health, cardiovascular disease, immunomodulation, thyroid function, and renal function [1]. The biological activities of soy foods have been linked to the content of isoflavone aglycones content in the soybeans (*Glycine max*). Two major isoflavone aglycones in soybeans namely genistein and daidzein were widely reported to have potential pharmacologic activity [2,3]. The previous study reported that contents of genistein and daidzein were estimated at about 50% and 40%, respectively, compared to the total soy isoflavones [4].

Tofu, one of soy food products prepared by coagulating soymilk followed by pressing the obtained curds into solid blocks, was well-known as a traditional food in Indonesia [5,6]. Tofu became more popular in Indonesia as a natural protein source and is widely produced due to the increasing demand in several areas in Indonesia [7,8]. However, there was limited publication reporting the content of both genistein and daidzein in tofu. It has become more important since the need for information regarding the intake levels of soybeans food products as well as the nutrients contents should be reported to consumers [9].

Reversed phase high-performance liquid chromatography (RP-HPLC) was reported in several studies to analyze the content of analytes in the mixture matrix [10,11]. Previous studies on analyzing soybean products were performed by RP-HPLC [12,13]. However, the appropriate RP-HPLC conditions should be evaluated to achieve the good separation between analytes. Response surface methodology (RSM), an experimental design for optimization purposes, can be applied according to the Box-Behnken design (BBD) for natural product research [14]. RP-HPLC conditions can be optimized computationally to predict the

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desirable conditions for compounds separation. The desirability functions generated from the desirability analysis stage can be employed to enhance the prediction quality of experimental design research [15]. This study aimed to develop an RP-HPLC method aided by the RSM for obtaining appropriate chromatographic condition for simultaneous analysis of genistein and daidzein in tofu samples. Experimental factors including methanol composition, flowrate, and column temperature were evaluated in this study in order to achieve several responses namely retention time, resolution, and tailing factor.

## 2. RESULTS

The experimental design for optimization of independent variables and dependent variables of genistein and daidzein separation using RP-HPLC was presented in Table 1.

**Table 1.** The BBD for optimization of independent variables and experimental dependent variables of genistein and daidzein separation using RP-HPLC

Run	Independent variables			Dependent variables					
				Genistein			Daidzein		
	X1	X2	X2	Y1g	Y2g	Y3g	Y1d	Y2d	Y3d
1	60	0.6	40	13.288	5.774	1.297	9.913	1.381	1.348
2	80	0.6	40	6.195	1.724	1.484	5.671	0.006	1.455
3	60	1.0	40	7.538	6.027	1.267	5.704	7.172	1.260
4	80	1.0	40	3.654	1.582	1.356	3.356	1.206	1.385
5	60	0.8	30	10.165	6.140	0.975	7.557	1.130	1.057
6	80	0.8	30	4.649	1.780	1.274	4.239	0.144	1.315
7	60	0.8	50	7.902	4.595	1.047	6.219	4.578	1.164
8	80	0.8	50	4.224	1.254	0	3.942	2.359	0
9	70	0.6	30	8.406	3.859	1.206	6.999	6.134	1.259
10	70	1.0	30	5.055	3.614	1.185	4.205	4.998	1.230
11	70	0.6	50	7.241	2.745	1.184	6.304	2.741	1.247
12	70	1.0	50	4.180	2.395	1.212	3.667	1.533	1.265
13	70	0.8	40	5.660	3.019	1.223	4.852	1.214	1.259
14	70	0.8	40	5.687	3.051	1.211	4.872	2.758	1.252
15	70	0.8	40	5.697	3.062	1.207	4.868	1.528	1.248
16	70	0.8	40	5.715	3.046	1.212	4.896	1.484	1.242

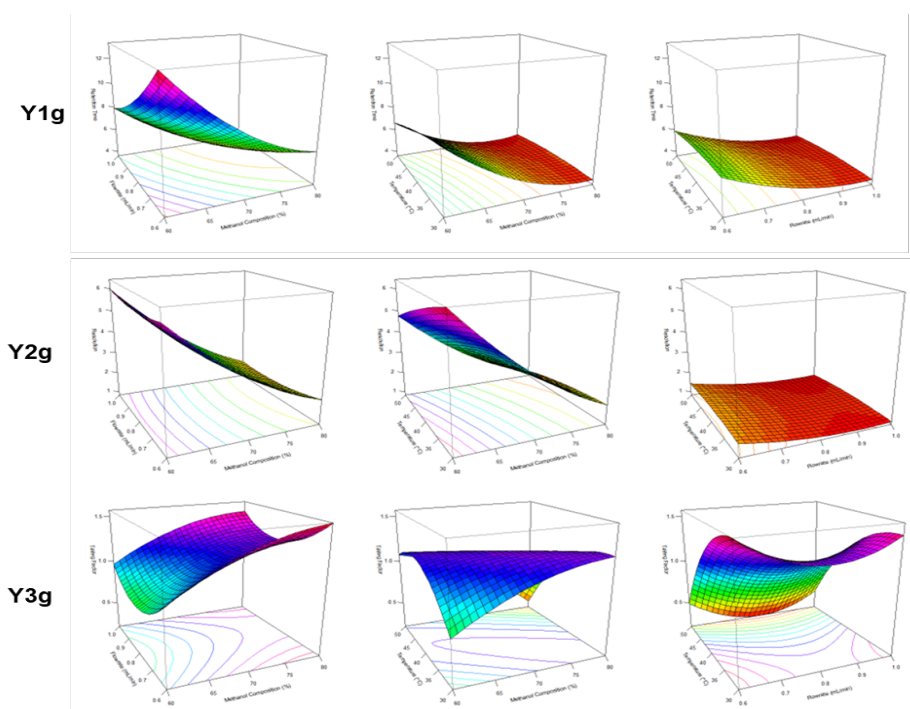
Notes: X1: methanol composition (%); X2: flowrate (mL.min<sup>-1</sup>); column temperature (°C); Y1g: retention time of genistein (min); Y2g: resolution of genistein; Y3g: tailing factor of genistein; Y1d: retention time of daidzein (min); Y2d: resolution of daidzein; Y3d: tailing factor of daidzein.

Sixteen experimental runs were carried out and observed. Retention time, resolution, and tailing factor of genistein and daidzein were evaluated. Each response for each analyte was modelled to obtain RSM model equations along with RSM properties such as multiple R<sup>2</sup>, adjusted R<sup>2</sup>, and p-value. The RSM model equations of retention time, resolution, and tailing factor for genistein and daidzein were presented in Table 2.

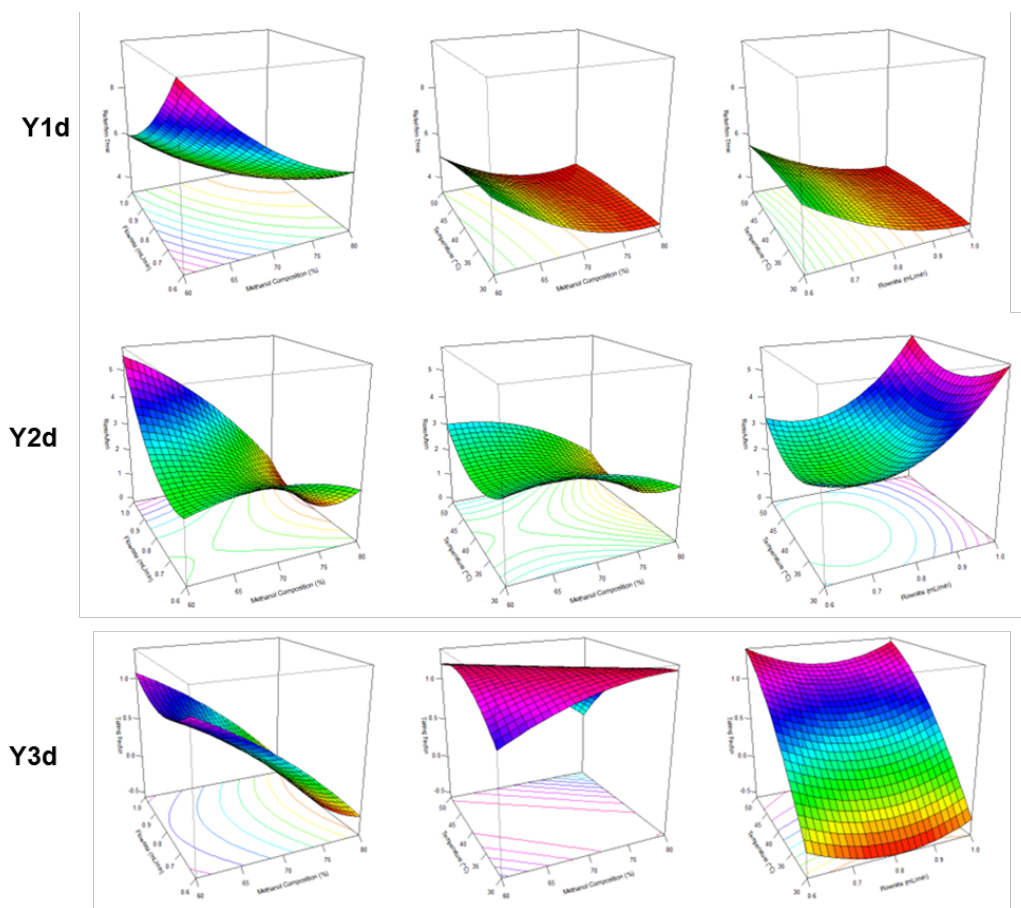
**Table 2.** The RSM model equations of retention time, resolution, and tailing factor for genistein and daidzein

Responses	RSM model equations	Multiple R <sup>2</sup>	Adjusted R <sup>2</sup>	p-value
<b>Genistein</b>				
Retention	$Y1g = 139.127 - 2.502X_1 - 68.008X_2 - 0.249X_3 + 0.401X_1X_2 + 0.005X_1X_3 + 0.036X_2X_3 + 0.012X_1^2 + 18.306X_2^2 - 0.002X_3^2$	0.9905	0.9761	2.274e-05
Resolution	$Y2g = 50.369 - 0.980X_1 - 5.186X_2 - 0.133X_3 - 0.049X_1X_2 - 0.003X_1X_3 - 0.013X_2X_3 + 0.005X_1^2 + 5.541X_2^2 - 0.001X_3^2$	0.9959	0.9898	1.794e-06
Tailing factor	$Y3g = -13.633 + 0.303X_1 - 9.692X_2 + 0.433X_3 - 0.012X_1X_2 - 0.003X_1X_3 + 0.006X_2X_3 - 0.001X_1^2 + 6.381X_2^2 - 0.003X_3^2$	0.8013	0.5032	0.1207
<b>Daidzein</b>				
Retention	$Y1d = 87.237 - 1.485X_1 - 46.699X_2 - 0.134X_3 + 0.237X_1X_2 + 0.003X_1X_3 + 0.020X_2X_3 + 0.007X_1^2 + 13.669X_2^2 - 0.001X_3^2$	0.9931	0.9826	8.813e-06
Resolution	$Y2d = -25.156 + 1.223X_1 - 6.444X_2 - 0.479X_3 - 0.574X_1X_2 - 0.003X_1X_3 - 0.009X_2X_3 - 0.006X_1^2 + 31.175X_2^2 - 0.009X_3^2$	0.4908	-0.2731	0.7358
Tailing factor	$Y3d = -13.358 + 0.310X_1 - 10.058X_2 + 0.421X_3 + 0.002X_1X_2 - 0.004X_1X_3 + 0.006X_2X_3 - 0.001X_1^2 + 5.975X_2^2 - 0.002X_3^2$	0.7811	0.4528	0.1519

According to the results, RSM model of Y1g and Y2g were analysed further for generating desirability function. Response surface plot of retention time (Y1g), resolution (Y2g), and tailing factor (Y3g) for genistein were depicted in Figure 1. Response surface plot of retention time (Y1d), resolution (Y2d), and tailing factor (Y3d) for daidzein were depicted in Figure 2.



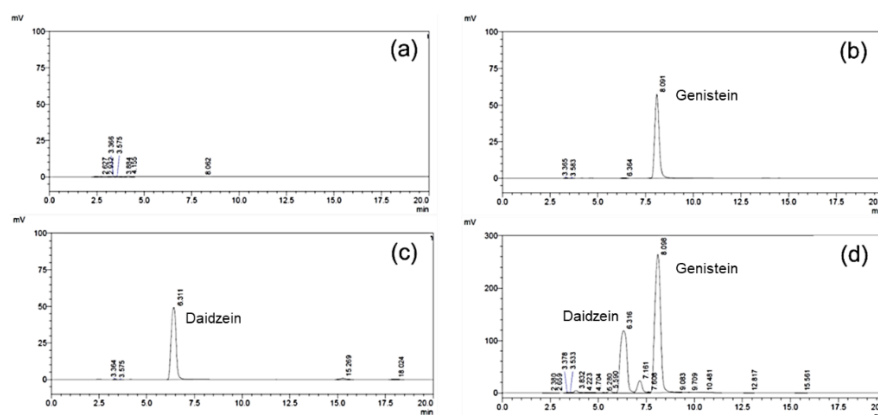
**Figure 1.** Response surface plot of retention time (Y1g), resolution (Y2g), and tailing factor (Y3g) for genistein



**Figure 2.** Response surface plot of retention time (Y1d), resolution (Y2d), and tailing factor (Y3d) for daidzein

The desirability functions have been generated using R statistical software with the package of ‘rsm’. Genistein retention time was set for a target value of 8 minutes, with the lower and upper value estimation of 6.5 and 10 minutes, respectively. Genistein resolution was set for a maximum value of 4.5, with a lower value estimation of 1.5. Daidzein retention time were set for a minimum value of 6.5 minutes, with the upper value estimation of 9 minutes.

The recommended conditions obtained from the RSM model followed by the desirability function were set in the HPLC system and applied for evaluating the solvent of methanol, genistein standard, daidzein standard, and tofu sample containing genistein and daidzein (Figure 3).



**Figure 3.** HPLC chromatograms of solvent (a), standard of genistein (b), standard of daidzein (c), and tofu sample containing genistein and daidzein (d). Mobile phase: methanol-water (60:40 v/v). Flowrate: 0.80 mL/min. Column: C<sub>18</sub> column of Hibar® 250-4,6 Purospher® STAR RP-18 endcapped (5 µm). Column temperature: 50°C. Wavelength detection at 260 nm. Volume injection: 10 µL.

**Table 3.** Results of system suitability test (n=6)

Analytes	Retention time		Area		Resolution	Tailing factor	Theoretical plates number
	Mean	RSD (%)	Mean	RSD (%)			
<i>Standards</i>							
Genistein	8.075	0.817	904670.8	0.734	4.403	1.201	5761.747
Daidzein	6.364	0.777	900766.0	0.915	4.664	1.220	3919.482
<i>Samples (tofu)</i>							
Genistein	8.047	0.713	5589872	0.910	4.185	1.255	5475.179
Daidzein	6.367	0.826	2823239	0.972	4.621	1.188	4007.568

Separation profiles of genistein and daidzein both in standard and sample solutions were observed. Six replications of standard solution and sample solution were injected into the HPLC system. Results of system suitability test were presented in Table 3.

### 3. DISCUSSION

#### 3.1. Experimental design

In this study, an optimization of the RP-HPLC condition for separating genistein and daidzein in tofu was performed. The experimental design was developed according to the BBD model with the implementation of RSM. Three independent variables or factors including methanol composition, flowrate, and column temperature were observed in this study. Retention time, resolution, and tailing factor of genistein and daidzein were stated as the dependent variables or responses. The BBD model was generated using R statistical software. A model of BBD using three factors, three levels, and four central points was successfully generated. This model was applied to optimize the RP-HPLC conditions followed by response observation to generate RSM model for each response.

#### 3.2. RSM observation

The RSM model for genistein was successfully generated for retention time (Y1g), resolution (Y2g), and tailing factor (Y3g). The quality of the RSM model of genistein can be evaluated according to multiple determination coefficient ( $R^2$ ), adjusted  $R^2$ , and p-value. It can be stated that experimental factors have significantly affected the responses only if the multiple  $R^2 \geq 0.8$  and adjusted  $R^2 > 0.8$ . Furthermore, the minimum difference between multiple  $R^2$  and the adjusted  $R^2$  (less than 0.2) indicates that the second-order polynomial models satisfactorily fit the actual data [16]. The p-value of the model indicates a good predictive model with a value of  $\leq 0.05$  [17]. It was found that the multiple  $R^2$  and adjusted  $R^2$  for Y1g were 0.9905 and 0.9761 (p-value =  $2.274 \times 10^{-5}$ ), respectively. The multiple  $R^2$  and adjusted  $R^2$  for Y2g were 0.9959 and 0.9898 (p-value =  $1.794 \times 10^{-6}$ ), respectively.

Similar to the genistein models, the RSM model for daidzein was successfully generated for retention time (Y1d), resolution (Y2d), and tailing factor (Y3d). It was found that the multiple  $R^2$  and adjusted  $R^2$  for Y1d were 0.9931 and 0.9826 (p-value =  $8.813 \times 10^{-5}$ ), respectively. Since only the retention time model of daidzein met the requirements of multiple  $R^2$ , adjusted  $R^2$ , and p-value; this RSM model was analysed further for generating desirability function.

#### 3.3. Desirability analysis

RSM can be developed along with the desirability analysis to obtain the selected condition for optimization purposes. Multiple responses of RSM with the significant model can be selected for desirability consideration. In this study, genistein retention time, genistein resolution, and daidzein retention time were chosen and applied in the desirability analysis.

The composite desirability was calculated computationally. It was found that the model predicted a total desirability of 0.9778 can be achieved by applying the methanol composition of 60%, flowrate of  $0.80 \text{ mL} \cdot \text{min}^{-1}$ , and column temperature of  $50^\circ\text{C}$ . Desirability values resulting from the desirability functions lie between 0 and 1. The desirability value of 0 corresponds to the undesirable response obtained from the predictive factors. On the other hand, the desirability value of 1 corresponds to the most expected responses [18,19].

#### 3.4. System suitability test

HPLC separation properties including retention time, area, resolution, tailing factor, and theoretical plates number were evaluated to ensure the appropriateness of the analytical method. According to the results, it can be found that the optimized HPLC conditions met the acceptance criteria for the system suitability test with minimum RSD of retention time and area ( $RSD < 1.0\%$ ), resolution of more than 4.0 ( $R_s > 2.0$ ), tailing factor of less than 2.0 ( $TF \leq 2.0$ ), and theoretical plates number of more than 3900 ( $N > 2000$ ) [20].

#### 4. CONCLUSION

An analytical method of RP-HPLC for simultaneously separating genistein and daidzein has been successfully developed. Optimization has been performed by applying the response surface methodology of the Box-Behnken design. The desirability functions have been successfully generated to strengthen the quality of the RSM. It was found that the optimized HPLC conditions were methanol composition of 60%, flowrate of 0.80 mL.min<sup>-1</sup>, and column temperature of 50°C. These conditions were set for the HPLC system followed by the system suitability test. Several separation properties such as retention time, area, resolution, tailing factor, and theoretical plates number were reported to meet the acceptance criteria of the system suitability test.

However, the optimized analytical method can be developed further. In the future, it is recommended to perform the analytical method validation to empirically demonstrate if the method is appropriate to be applied for the intended purposes.

#### 5. MATERIALS AND METHODS

##### 5.1. Materials

Tofu sample was purchased from the local market in Yogyakarta, Indonesia. Reference standards of genistein and daidzein were purchased from Sigma-Aldrich. Solvents of methanol gradient grade for liquid chromatography (Merckmillipore), ethyl acetate, petroleum ether (Smart Lab), and redistilled water (PT. Ikapharmindo Putramas) were used in this study. Anhydrous Na<sub>2</sub>SO<sub>4</sub> was purchased from Merckmillipore.

##### 5.2. Instrumentation and Software

A system of HPLC Shimadzu® LC-2010 CHT with UV/Vis detector accompanied with a C18 column of Hibar® 250-4,6 Purospher® STAR RP-18 endcapped (5 µm) was used in this study. Other instrumentation were listed as follow: a system of Buchi Rotary Evaporator ultra-micro analytical balance RADWAG® series of UYA 2.3Y (max: 2.1 g, min 0.01 mg), Gast® vacuum pump, Retsch® T460 ultrasonicator, sterile syringe filter with a 0.2 µm pore size hydrophilic PTFE membrane (Merckmillipore), and a set of Socorex® micropipettes. The R statistical software version 4.2.0 along with R Studio software version 2022.12.0 Build 353 were exploited in this study. The R software package namely 'rsm' was installed and applied to carry out statistical analysis of RSM and desirability analysis.

##### 5.3. Methods

###### 5.3.1. Standard and sample preparation

An accurate weight of 5.0 mg for each genistein and daidzein standard were transferred into 10 mL volumetric flask. Genistein and daidzein standards of each volumetric flask were diluted in methanol into the volume. These solutions were filtered using sterile syringe filter membrane before injection into HPLC system.

Preparation of tofu sample was applied using a modification from Yuliani et al. (2016) [2]. One kg of tofu was mixed and macerated in 50 mL petroleum ether for 40 minutes at 150 rpm and the petroleum ether was subsequently removed. The obtained residue and hydrophilic phase were fractionated using ethyl acetate and water. The fraction of ethyl acetate was separated and filtered to eliminate the solid residue. The ethyl acetate fraction was added with sodium sulfate anhydrous in order to remove the water. The obtained yellowish solution was subsequently proceeded using rotary evaporator to remove the solvent. The remained residue was diluted using methanol. This solution was filtered using sterile syringe filter membrane before injection into HPLC system.

###### 5.3.2. Experimental design

The BBD of using three factors, three levels, and four central points was developed. The percentage of methanol (X1), flowrate (X2), and column temperature (X3) was selected as factors (independent variables).

On the other hand, separation properties such as retention time (Y1), resolution (Y2), and tailing factor (Y3) were stated as the responses (dependent variables). Observational independent variables along with the experimental levels were presented in Table 4.

**Table 4.** Observational independent variables to build the BBD model

Variables	Levels		
	Low	Medium	High
X1: methanol composition (%)	60	70	80
X2: flowrate (mL.min <sup>-1</sup> )	0.6	0.8	1.0
X3: column temperature (°C)	30	40	50

Note: \* cross validation was performed using leave one out technique

Sixteen experimental runs will be achieved since the number of experiments can be calculated using formula  $2k(k-1)+C_p$ , where k is the number of factors and  $C_p$  is the number of central points. These runs were executed using the RP-HPLC system at 260 nm UV detection and volume injection of 10  $\mu$ L.

### 5.3.3. RSM observation

Sixteen BBD runs generated from the software were executed and observed. All responses for each compound were recorded and listed to build the RSM model. All factors and responses were exploited for generating the second-order polynomial models. The estimated coefficients of the RSM model were considered to obtain the following formula:

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 \dots (1)$$

where, Y is the predicted response,  $X_1$ ,  $X_2$ ,  $X_3$  are the independent variables,  $\beta_0$  is the intercept,  $\beta_1$ ,  $\beta_2$ ,  $\beta_3$  are the linear effect,  $\beta_{12}$ ,  $\beta_{13}$ ,  $\beta_{23}$  are the interaction effect, and  $\beta_1^2$ ,  $\beta_2^2$ ,  $\beta_3^2$  are the quadratic effect.

After achieving all functions for each compound, significant models were selected for generating the desirability function in the desirability analysis stage of the research. The perspective plots for each response were also depicted to visualize the RSM models.

### 5.3.4. Desirability analysis

The desirability function has been generated according to the previous study [21]. Each response can be set for minimum, maximum, or specific target value along with upper and lower value estimation.

### 5.3.5. System suitability test

The system suitability test was performed by injecting standards and samples solution containing genistein and daidzein. These solutions were filtered using sterile syringe filter membrane before injection into HPLC system. These solutions were injected in six replications.

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**Author contributions:** Concept - F.D.O.R.; Design - F.D.O.R.; Supervision - F.D.O.R.; Resources - M.R.G., D.C.A.P.; Materials - M.R.G., D.C.A.P.; Data Collection and/or Processing - F.D.O.R.; Analysis and/or Interpretation - F.D.O.R.; Literature Search - D.C.A.P.; Writing - F.D.O.R., M.R.G.; Critical Reviews - F.D.O.R., M.R.G., D.C.A.P.

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