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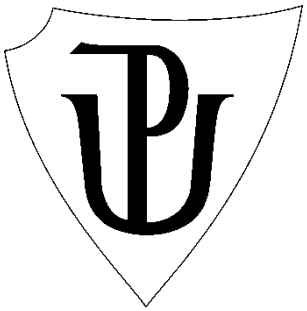

FORENSIC APPLICATIONS OF MASS SPECTROMETRY

DOCTORAL THESIS

Author: Volodymyr Pauk

Field of study: Analytical Chemistry

Supervisor: prof. RNDr. Karel Lemr, Ph.D.

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I hereby declare that I have written the dissertation thesis myself. All used information and literary sources are indicated in the references.

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Acknowledgement

I am grateful to my family for their support and to my wife for inspiration through my entire education.

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SUMMARY

This Ph.D. thesis is devoted to application of mass spectrometry in the field of forensic science. Mass spectrometry alone or in combination with separation methods, such as UHPLC and UHPSFC, is a versatile tool that allows fast and efficient analysis of various analytes. It is one of the most desired techniques in the field of forensic science due to its high selectivity and sensitivity. However, a mass spectrometer should not be considered as a "black box" where a sample is loaded and a finished result is dropped out. Preliminary laborious method development including sample treatment that takes into account specificity of analytes should be carried out. Understanding of physical principles of instrument operation as well as chemical properties of studied substances is needed. Only then reliable methods suitable for routine application can be developed.

Therefore, the main purpose of this work is development of new mass spectrometric methods useful for identification of specific substances in objects within the scope of forensic science. The thesis focuses on the following three topics: detection of pigments in oil paintings, identification of binders in water-soluble painting medium and analysis of new designer drugs. The information obtained can be useful for approximate dating and evaluation of authenticity/origin of historical artifacts and artworks or beneficial for characterization of composition of tablets, pills, powders sold as "legal highs".

Theoretical part covers general forensic applications of mass spectrometry as well as specific knowledge about each of the mentioned topics. First, information on the objects of analysis is given (e.g. historical pigments, plant gums, new designer drugs). Second, existing analytical methods and protocols are described. Third, problematic issues are discussed and original tasks for own work are defined.

Experimental and Results and Discussion parts are divided into three sections corresponding to each topic. The first one deals with the identification of historical pigments indigo and Prussian blue in oil paintings. Analysis is based on simple chemical reactions, hydrolysis of Prussian blue and reduction of indigotine in alkaline environment, leading to efficient dissolving and allowing sensitive detection of the aforementioned pigments. The developed FIA/ESI-MS method is fast and makes possible identification of both inorganic and organic components without chromatographic separation. Calculated limits of detection are at picogram levels. Potential of the developed method was proven in analysis of blue

samples from two oil paintings (20th century) and a microsample from the painting of 'Crucifixion', St. Šebestián church on St. Hill, Mikulov, Czech Republic.

The second section deals with differentiation between plant gums used as binders in historical and art objects. Plant-derived polysaccharides are decomposed prior the analysis by microwave-assisted hydrolysis. Hydrolysates containing monosaccharides in different ratios specific to each type of plant gum are analyzed by means of supercritical fluid chromatography hyphenated to mass spectrometry. Subsequently, chromatographic data is subjected to principal component analysis which reveals differences in composition between plant gums. Samples of high-grade aquarelle paints and one archaeological sample were analyzed and compared with profiles of the three most widespread plant glums (gum Arabic, cherry gum and gum tragacanth).

The third section describes development of a new method for analysis of modern synthetic drugs of abuse, so-called "new designer drugs", belonging to classes of cathinones and phenylethylamines. The analysis is based on supercritical fluid chromatography with mass spectrometric detection. Efficient separation of four isomeric pairs and most of remaining analytes (fifteen compounds in total) was achieved in less than 3 minutes on BEH (silica) and Fluoro-phenyl stationary phases with appropriate mobile phase modifiers. Electrospray ionization with a triple quadrupole analyzer in a selected reaction monitoring mode provided an additional dimension for differentiation and sensitive detection of all investigated substances.

Therefore, the defined tasks were successfully fulfilled. The developed mass spectrometric methods have proven their suitability for forensic-related applications. Part of the results has been published in impacted analytical journals.

SOUHRN

Disertační práce se zabývá aplikacemi hmotnostní spektrometrie ve forenzní vědě. Hmotnostní spektrometrie samotná nebo ve spojení se separačními metodami jako UHPLC a UHPSFC představuje poměrně všestranný nástroj, který umožňuje rychlou a účinnou analýzu různých analytů. Je to jedna z nejžádanějších technik v oblasti forenzní vědy z důvodu její vysoké selektivity a citlivosti. Hmotnostní spektrometr však nesmí být považován za „automatický stroj“, kam se vzorek vloží a odkud vypadne hotový výsledek. Musí se provést často pracný vývoj metody zahrnující úpravu vzorku, který počítá se specifikou analytů. K tomu je nutné pochopení fyzikálních principů chodu přístroje stejně jako chemických vlastnosti studovaných látek. Teprve potom mohou být vyvinuty spolehlivé metody vhodné pro rutinní použití.

Hlavním cílem této práce je vývoj nových metod na bázi hmotnostní spektrometrie pro identifikaci specifických látek v objektech spadajících do oblasti zájmu forenzní vědy. Disertace se zaměřuje na tři témata: detekce pigmentů v olejových malbách, identifikace pojiv ve vodorozpustných malířských barvách a analýzu nových syntetických drog. Získané informace mohou být využity pro přibližné datování a hodnocení pravostí/původu historických artefaktů a uměleckých děl nebo pro charakterizaci složení tablet, pilulek, prášků zneužívaných jako drogy.

Teoretická část pokrývá obecné forenzní aplikace hmotnostní spektrometrie a specifické informace ohledně každého ze zmíněných témat. Zaprvé jsou shrnuty informace o objektech analýzy (historické pigmenty, rostlinné pryskyřice, nové syntetické drogy). Zadruhé jsou popsány stávající analytické metody a protokoly. Zatřetí jsou diskutovány možné problémy analýz a definovány původní úkoly pro vlastní práci.

Kapitoly Experimentální část a Výsledky a diskuse jsou rozděleny do tří oddílů, které odpovídají jednotlivým tématům. První oddíl pojednává o identifikaci historických pigmentů indiga a Pruské modři v olejových malbách. Analýza je založena na jednoduchých chemických reakcích (hydrolýza Pruské modři a redukce indigotinu v alkalickém prostředí) umožňujících rozpuštění pigmentů a zároveň dovolujících jejich citlivou detekci. Vyvinutá FIA/ESI-MS metoda je rychlá a umožňuje identifikaci obou látek bez chromatografické separace. Zjištěné meze detekce jsou na úrovni pikogramů. Možnosti této metody byly prokázány při analýze vzorků modré barvy ze dvou olejových maleb (počátek 20. století) a mikrovzorku z obrazu „Ukřižování“, kostel Sv. Šebestiána na Sv. Kopečku v Mikulově.

Druhý oddíl je zaměřen na rozpoznání rostlinných gum používaných jako pojiva v historických a uměleckých objektech. Rostlinné polysacharidy se před analýzou hydrolyzují pomocí mikrovlnného záření. Hydrolyzáty obsahující monosacharidy v různých poměrech, specifických pro každý typ gumy, jsou analyzovány pomocí superkritické fluidní chromatografie s hmotnostní detekcí. Následně jsou experimentální data zpracována metodou hlavních komponent (PCA), která je schopna odhalit rozdíly ve složení jednotlivých gum. Vzorky akvarelů vysoké kvality a jeden archeologický vzorek byly analyzovány a srovnány s profily třech nejčastěji používaných přírodních gum (Arabská guma, třešňová guma a tragant).

Třetí oddíl popisuje vývoj metody pro analýzu nových syntetických drog, tzv. "new designer drugs", patřících do skupin katinonů a fenylethylaminů. Analýza je založena na separaci pomocí superkritické fluidní chromatografie s hmotnostní detekcí. Použití stacionárních fází BEH (silikagel) a Fluoro-phenyl s vhodnými modifikátory v mobilní fázi umožňuje separaci většiny z patnácti studovaných návykových látek, především čtyř párů isomerických sloučenin, méně než za tři minuty. Ionizace elektrosprejem ve spojení s trojitým kvadrupólem v SRM módu poskytuje další dimenzi pro rozlišení a citlivou detekci všech sledovaných látek.

Kladené úkoly byly úspěšně splněny. Vyvinuté hmotnostně-spektrometrické metody prokázaly svou účlenost pro forenzní aplikace. Část výsledků byla publikována v impaktovaných analytických časopisech.

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1 INTRODUCTION

Forensic science is situated at the intersection of biological, chemical, physical sciences and criminal justice. This interdisciplinary field deals not only with controlled substances, arson investigation or detection of explosives but also answers questions of authenticity, provenance and age of various valuable objects. Investigation of forgeries, fakes and copies of written documents, jewelry, historical and artistic objects is an important research area within the framework of forensic science. Powerful, sensitive and selective analytical techniques which offer all necessary information are required to address the forensic challenges.

Mass spectrometry (MS) is one of such tools that fulfill imposed requirements. Its importance cannot be overemphasized since it is the most versatile detection technique which provides structural information and identification of unknowns [1]. It can be used standalone and in combination with separation techniques in variety of applications. Electrospray (ESI) is the most widespread ionization source, suitable for a broad range of compounds from polar to ionic, such as investigated in this study. Various types of mass analyzers are available. Modern triple quadrupoles offer wide dynamic range, high sensitivity in selected reaction monitoring (SRM) mode and fast scan speed. These are preferred machines for quantitation purposes. Hybrid instruments, such as quadrupole-time of flight (Q-TOF) and linear ion trap-orbitrap, achieve high resolution, are capable of accurate mass measurement and allow MS/MS experiments. They are perfect for "fingerprint" profiling and identification of analytes in non-purified samples, such as painting medium. Of course, Fourier-transform ion cyclotron resonance (FT-ICR) spectrometers possess unbeaten resolution, but their price is directly proportional to the latter and maintenance costs are higher as well.

There is a huge bunch of existing protocols utilizing combination of MS and gas chromatography (GC), liquid chromatography (LC) or capillary electrophoresis (CE) in forensic science. Some of these procedures were established many years ago and are subjected to restrictions of outdated techniques. Since new instruments with higher resolution, sensitivity and efficiency are emerging, one can benefit from data-rich results, reduction of analysis time and lower required sample amount.

Supercritical fluid chromatography (SFC) has gained much interest in recent years [2]. It is a technique alternative to normal-phase chromatography, that offers speed of analysis and environmental benefits. Since its discovery SFC instrumentation has undergone a number of

improvements and now it can compete with ultra-high performance liquid chromatography (UHPLC) in terms of performance. Its range of potential analytes can be significantly extended by use of various mobile phase modifiers and additives making it complementary to reversed-phase LC or even hydrophilic interaction liquid chromatography (HILIC). It is useful for resolution of isomers that cannot be unambiguously distinguished by MS.

Provided with such a wide spectrum of instruments, development of analytical methods is the priority task of an analytical chemist. Without a proper setting this hardware would be just a tool without a master. Therefore, we focused our efforts on the development of new analytical protocols utilizing advantages of the mentioned techniques and instrumentation. We successfully used ESI as one of the most versatile ionization sources, although tested others, e.g. atmospheric pressure chemical ionization (APCI) and matrix-assisted laser desorption ionization (MALDI), and high-resolution Q-TOF instrument for identification of pigments in complex oil painting matrix and a triple quadrupole analyzer for analysis of saccharides in binding medium and new designer drugs (NDDs). The last two applications dealt with isomeric compounds and required their preliminary separation by a chromatographic technique. Utilization of UHPSFC system allowed development of fast and sensitive methods.

2 THEORETICAL PART

2.1 Application of mass spectrometry in forensic science

Mass spectrometry has found wide utilization in forensic science because of its high sensitivity and selectivity. Efficiency of mass spectrometry is further increased by hyphenation with separation techniques, such as GC, CE, LC or SFC. Its most important areas of application in forensic science framework include:

- forensic toxicology and examination of controlled substances – analysis of drugs of abuse and relevant compounds (NDDs, antidepressants, neuroleptics, hypnotics etc.), doping control, investigation of natural poisons [3-6];
- trace evidence – analysis of arson and explosive residues, chemical warfare agents, investigation of hair, fibers, paint, [4,6];
- investigation of copies, fakes and forgeries – evaluation of currency, jewelry, artworks, historical artifacts, written documents (inks and paper) [4,7];
- food adulteration – detection of illicit food additives and dyes, differentiation of synthetic and biogenic origin [6].

As the technical progress goes further and new analytical possibilities emerge, forensic science follows these changes. In the very recent review on developments in forensic mass spectrometry several trends were outlined [8]. Field-portable mass spectrometers have high potential value for evidence collection at a crime scene. Ambient ionization techniques, e.g. desorption electrospray, direct analysis in real time (DART) are continuously gaining interest in forensic applications over the past decade since they allow direct sampling and real-time monitoring of analytes. Some of these ionization techniques as well as laser desorption methods have spatial resolution sufficient for imaging of a chemical "signature" in fingerprints or hair. Isotope ratio measurement combined with sophisticated chemometric methods has become routine in forensic laboratories and provides information on provenance of drugs, explosives, petroleum, food products or helps to distinguish their natural or synthetic origin.

2.2 Identification of Prussian blue and indigo by FIA/ESI-MS

Part of materials presented in this section has been published in the following articles:

V. Pauk, V. Havlíček, B. Papoušková, P. Sulovský, K. Lemr, Simultaneous identification of historical pigments Prussian blue and indigo in paintings by electrospray mass spectrometry, *J. Mass. Spectrom.* 48 (2013) 927–930 [9].

V. Pauk, P. Barták, K. Lemr, Characterization of natural organic colorants in historical and art objects by high-performance liquid chromatography, *J Sep Sci.* 37 (2014) 3393–3410 [10].

2.2.1 Analysis of historical pigments and dyes

Identification of historical colorants provides crucial information for preservation and restoration of art objects. The analytical data gives useful clues on the painting technique of a craftsman or an artist as well as on household activity and the culture of a certain historical period. Since specific colorants were used during different times or in particular geographic locations, their identification assists in evaluation of object authenticity, dating or localizing the provenance of historical artifacts.

Colorants can be divided into two groups: dyes and pigments [11]. Dyes are mostly organic substances at least temporary soluble in the vehicle. Pigments are usually inorganic compounds (metal oxides, sulfides and other minerals) insoluble in the applied medium. A dye penetrates into the substrate, usually fibers of textile, whereas a pigment is bonded to the surface of the substrate and is usually applied as a suspension in a suitable binding medium (oils, proteins, gums, resins). Some colorants can be used in both ways, as dyes and as pigments. Examples are a vat dye indigo that was also used as a pigment in oil paintings [12] and drawings [13] or an insect-derived dye cochineal that after treatment with mordant (e.g. alum $KAl(SO_4)_2$) and addition of metal salt (like chalk $CaCO_3$) produces insoluble Ca-Al-colorant complex, carmine lake pigment [13].

A colorant itself can be a mixture of several substances. Compounds bearing a chromophore group are called coloring matters or coloring principles. Some dyes can contain the same coloring matters in different amounts. For example, kermesic acid is the main component of kermes dye and is also present in cochineal but at much lower level [14]. Moreover, the ratio of coloring matters in the same dyes derived from different species can vary greatly, e.g. alizarin, purpurin and munjistin in madder (*Rubia* sp.) [13] or carminic and kermesic acid in cochineal [14]. A dyestuff can also contain minor components specific for

particular species, like in case of sea snails. Some of these mollusks (*Bolinus brandaris* L., *Stramonita haemastoma* L.) produce dyes composed entirely of brominated indigo-derivatives, while dyes produced by the others (*Hexaplex trunculus* L.) contain some amount of non-brominated indigo [15,16]. Dyes can also contain degradation products and mineral impurities useful for identification purposes [17-19].

The available amount of a sample from an art or historical object is often extremely small, but information about main and minor components, corresponding degradation products as well as quantitative ratio of substances can be required to reach a final conclusion. This issue can be solved only by utilizing modern highly sensitive and selective analytical methods.

Various spectroscopic methods are potentially suitable for analysis of art objects, but each has some limitations [7,10]. Techniques such as spectroscopy in ultraviolet/visible region (UV/Vis) [20,21], infrared spectroscopy (IR) [20], Raman [22-26], fluorescence spectroscopy [27], X-ray fluorescence spectroscopy (XRF) [20,25] or nuclear magnetic resonance spectroscopy (NMR) [25] are virtually non-destructive, require no or little sample preparation, and most of them can be performed *in situ*. Nowadays multi-technique spectroscopic protocols are preferred [28,29]. However, UV/Vis spectroscopy is not as selective as other techniques and gives limited structural information. Fluorescence or spectral overlap of binders, pigments, and other components strongly affect Raman and IR spectra. Fluorescence spectroscopy requires the presence of fluorescent active compounds. XRF is able to detect only metal-containing colorants and is not suitable for identification of organic compounds. NMR is an excellent tool for identification of individual compounds but can be insufficiently sensitive for very small amounts of sample and is unsuitable for analysis of trace components in mixtures.

On the contrary, combination of separation techniques and mass spectrometry (CE/MS, GC/MS and HPLC/MS) is sufficiently sensitive and selective, provides structural information and allows identification of unknown compounds [30]. The disadvantage is that laborious sample treatment is often necessary. For instance, derivatization of the target analytes is a compulsory requirement for GC/MS [19], [31-34] and sample treatment for HPLC in some cases can last up to few hours [35]. Nevertheless, HPLC remains the method of choice for analysis of natural dyes since 1975 when it provided data for investigation of binding media in paintings [36] and, later, for dyestuffs [37].

Usually, only a very small amount of sample is taken (0.1–0.5 mg) since the integrity of an artifact must be maintained. Scrapes of paint or dissected fibers of a textile are further treated with a small volume (50–400 μl) of a proper solvent mixture under heating to extract the coloring matters. In some cases several consequent extractions may be applied if presence of different colorant classes is suspected. After evaporation extracts are redissolved, filtered or centrifuged and injected into HPLC system. Separated components are detected with UV/Vis (diode-array detector) or MS detectors. The latter are more favorable since not only lower limits of detection (LOD) are achieved but accurate mass measurement and MS/MS data supports the identification. Summary of published HPLC methods for analysis of natural organic colorants including sample treatment procedures is presented in **Tab. 2.1**. More information on this topic can be found in the author's review [10].

Direct MS methods without prior chromatographic separation are useful for fast analysis of insoluble pigments or when confirmation of presence of a certain colorant is needed. DART was utilized for *in situ* analysis of several dyes belonging to anthraquinoid, flavonoid and indigoid classes in dyed fibers [38]. Laser desorption ionization (LDI) was used for identification of shellfish purple on archeological ceramic fragments without previous sample treatment [39]. Secondary ion mass spectrometry was beneficial for surface analysis of organic as well as inorganic components and imaging of paint cross sections with high spatial resolution [40-42]. Despite the advantages of the mentioned direct MS techniques, they are not suitable for quantitative analysis and require dedicated instrumentation which can be expensive, additional maintenance and technically skilled operators.

Table 2.1 List of HPLC methods for analysis of natural colorants (adopted from [10] with modifications)

Ref.*	Investigated colorants[a)]	Extraction procedure	Column; dimensions, mm; particle. d, µm; temperature	Mobile phase	Time, min; flow, ml/min	Detection, LOD
1985 [26]	anthraquinoids (11)	3 M HCl, 100 ºC	Nucleosil C18; 150×4.1	A: water B: MeOH C: 1% form. acid	55; 1.5	UV/Vis
1989 [66]	anthraquinoids (insect reds) (11)	water/MeOH/37% HCl 1:1:2, hot; rediss. water/MeOH 1:1	-	-	-	UV/Vis
1989 [92]	anthraquinoids (madder) (1)	MeOH/20% form. acid; rediss. 20% DMF/MeOH	Cosmosil ODS 5C18; 150×4.6 Shim-pack CLC ODS(M); 150×4.6	MeOH/0.1 M amm. acetate/ac. acid 100:40:7 (isocratic)	24; 1	UV/Vis TS-MS, SIM
1992 [67]	anthraquinoids, flavonoids, indigo (27)	water/MeOH/37% HCl 1:1:2, 100 ºC; rediss. water/MeOH 1:1 pyridine, 100 ºC	Spherisorb ODS2, C18; 100×4.6; 3	A: water B: MeOH 10% C: 5% ph. acid	30; 1.2	UV/Vis
1994 [78],1995 [79, 80] 2005 [35]	anthraquinoids, shellfish purple, indigo (12)	3 M HCl/MeOH 1:1, 100 ºC; rediss. MeOH DMF, 150 ºC	Lichrosorb 15537 RP-18, C18; 150×4; 7	A: water B: MeOH 10% C: 5% ph. acid	20; 1	UV/Vis
1996 [91]	anthraquinoids, flavonoids, indigo, carthamin, tannins (38)	3 M HCl/MeOH, 100 ºC; rediss. water; MeOH	Spheri-5 ODS, C18; 100×2.1; 5; 38 ºC	A: water/0.1% TFA B: ACN/0.1% TFA	40; 0.6	UV/Vis
1997 [77]	shellfish purple (7)	DMF, hot	Kromasil; 250×4.6; 5	A: water B: MeOH 10% C: 5% ph. acid	23[b)]; 1	UV/Vis
1999 [62]	anthraquinoids (8); naphthoquinoids (3)	water/MeOH/37% HCl 1:1:2, 100 ºC	Purospher RP18e, C18; 125×4; 5	A: 0.1 M aq. citrate buffer B: MeOH MeOH/0.2 M aq. acetate buffer 72:25 (isocratic)	12[b)]; 0.6 10[b)]; 0.5	UV/Vis, 0.6-12ng
2003 [95]	anthraquinoids (insect reds) (4)	10% HCl, 65 ºC; ext. n-amyl alcohol, MeOH	Zorbax SB-C18; 250×4.6; 5	water/80% MeOH/ 0.2% ac. acid	12[b)]; 0.7	UV/Vis, 0.18-1.71 µg/ml; ESI-MS, 30-90 ng/ml

Table 2.1 Continued

2003 [85]	anthraquinoids, flavonoids, indigo (12)	3 M HCl/EtOH 1:1, 100 ºC; rediss. water/MeOH 1:1	Phenomenex Luna, C18; 250×4.6; 5; 40 ºC	A: water/5% ACN/0.1% TFA B: ACN/0.1% TFA	36[b)]	UV/Vis
	indigoids (4)	warm pyridine		A: water/ACN/THF 50:45:5 B: THF/ACN 95:5	18.3[b)]	
2003 [90]	anthraquinoids, flavonoids, indigoids (13)	3 M HCl/EtOH 1:1, 100 ºC; rediss. water/MeOH 1:1	Zorbax RX-C18; 150×2.1; 5; 30 ºC, 20 ºC	A: water/0.3% form. acid B: ACN	40; 0.2	UV/Vis; ESI-MS, APCI-MS, MRM
2004 [59]	insoluble reds (15)	water/MeOH/37% HCl 1:1:2, 100 ºC; rediss. DMF water/MeOH 1:1, 100 ºC DMF, 100 ºC	Hypersil BDS C18; 100×2.1; 3; 30 ºC	A: water B: ACN C: 1% MSA	32[b)]; 0.3	UV/Vis
2008 [60]	anthraquinoids, flavonoids, hydroxybenzoic acids (20)	water/MeOH/37% HCl 1:1:2, 100 ºC; rediss. DMSO	Hypersil BDS C18; 100×2.1; 3; 30 ºC	A: water B: ACN 10% C: 1% MSA	45; 0.3	UV/Vis, 3-24 ppb
				A: water B: MeOH 10% C: 1% MSA		FLD (ZrO^{2+}), 1-226 ppb
2004 [54]	indigoids (10)	DMSO/HCl 100:5, 80 ºC; ACN	Zorbax SB-C18; 150×4.6; 3.5	A: water/0.15% form. acid B: ACN	35; 0.6	UV/Vis; ESI-MS, SIM 0.01-0.15 µg/ml
2006 [50]	flavonoids, neoflavones, indigoids (16)	DMSO/37% HCl 100:2, 80 ºC; MeOH		A: water/0.15% form. acid B: MeOH	55; 0.5	UV/Vis; ESI-MS, SIM
2011 [39]	anthraquinoids, flavonoids, indigoids (29)	MeOH/form. acid 9:1, 60 ºC; MeOH/37% HCl 7:1, 60 ºC	Zorbax SB-C18; 150×4.6; 3.5	A: water B: MeOH 10% C: 1% form. acid	27; 0.5	UV/Vis; ESI-MS, SIM
2004 [55]	anthraquinoids, indigoids	MeOH/30% HCl 30:1, 60 ºC; ext. ethyl acetate	Genesis 80, C18; 250×4.6	A: water/19% ACN/ 0.1% TFA B: water/50% ACN	40; 1	UV/Vis; FLD
2005 [68] 2009 [49]	anthraquinoids, flavonoids (4); flavonoid glycosides	water/MeOH/37% HCl 1:1:2, 100 ºC; rediss. water/MeOH 1:1 MeOH/form. acid 95:5, 40 ºC; rediss. water/MeOH 1:1 0.001 M EDTA/ ACN/MeOH 2:10:88, 60 ºC; rediss. water/MeOH 1:1	Vydac 214TP52, C4; 250×2.1; 5	A: water B: ACN 5% C: 1% form. acid	60; 0.2	UV/Vis; ESI-MS
2006 [86]	shellfish purple (10)	DMSO, 100 ºC	Symmetry C18; 150×3; 5	A: water B: MeOH 10% C: 5% ph. acid	30; 0.8	UV/Vis, 50 pg
2008 [31, 88]					25; 0.8-2.1	UV/Vis
2006 [81]	shellfish purple (6)	DMF, 70 ºC	XTerra C18, 40 ºC; 250×3; 5	A: water/0.001% TFA B: ACN/0.001% TFA	12[b)]; 0.4	UV/Vis; APCI-MS, SIM

Table 2.1 Continued

2006 [63]	anthraquinoids, flavonoids, indigoids (21)	water/MeOH/37% HCl 1:1:2, 100 ºC; rediss. DMF, water/MeOH 1:2	-	-	32.5[b)]	UV/Vis; ESI-MS, SIM 0.01 µg/ml
2006 [61]	anthraquinoids, flavonoids, indigotin (11)	water/MeOH:37% HCl 1:1:2, 100 ºC; rediss. water/MeOH 1:1; MeOH/DMF 1:1, 100 ºC	Phenomenex Sphericlone, C18; 150×4.6; 5; 25 ºC Phenomenex Gemini, C18; 150×2.1; 5	A: 20% MeOH B: MeOH 10% C: 5% ph. acid	22.5; 1.2 25; 0.35	UV/Vis
2006 [57]	anthraquinoids, flavonoids, indigotin (6)	0.1 M SDS	Phenomenex Luna, NH_2; 250×4.6; 5; 35ºC	A: 40 mM SDS/10 mM ph. buffer/0.1% TFA B: ACN	42[b)]; 0.6	UV/Vis
2007 [5]	anthraquinoids, flavonoids, hydroxybenzoic acids (17)	water/MeOH/37% HCl 1:1:2, 100 ºC; rediss. water/MeOH 1:1	Zorbax RX C8; 150×2.1; 5	A: water/0.3% form. acid B: ACN	45; 0.2	UV/Vis, 2-31 ng/ml; ESI-MS, SIM, MRM, 0.5-32 ng/ml
2011 [6]	anthraquinoids, tannins, flavonoids, hydroxybenzoic acids, indigotin (15)	water/MeOH/37% HCl 1:1:2, 100 ºC; rediss. DMSO	Zorbax RX C8; 150×2.1; 5; 30 ºC	A: water/0.3% form. acid B: ACN	32[b)]; 0.2	UV/Vis, 10-70 ng/ml; APCI-,ESI-MS SIM, MRM 0.4-20 ng/ml
2008 [45]	anthraquinoids, flavonoids and their glycosides	ACN/MeOH/4 M HF 1:1:2 water/MeOH/12 M HCl 1:1:2, 110 ºC; rediss. water/MeOH 1:1	Alltima RP, C18; 250×4.6; 5; 20 ºC	A: MeOH B: water/5% ACN C: 0.1% TFA/MeOH 1:1 D: ACN	65; 1	UV/Vis
2008 [47]	anthraquinoids, flavonoids, indigotin, indirubin (12)	DMF, 80 ºC; water/MeOH/37% HCl 1:1:2 100 ºC; rediss. DMF, DMSO	Alltima HP, C18, 35 ºC; 250×3; 5; 33ºC	A: water/0.1% TFA B: ACN/0.1% TFA	35; 0.5	UV/Vis, 2-29 ng/ml
2009 [46]						
2009 [37]	+flavonoid glycosides, curcuminoids (14)	water/MeOH/0.5 M citric acid 1:1:1, 100 ºC; DMSO water/MeOH/1 M ox. acid 1:1:1, 100 ºC; DMSO water/MeOH/2 M TFA 1:1:1, 100 ºC; DMSO 5 M form. acid / water/MeOH/0.5 mM EDTA 2:1:1:4, 100 ºC; DMSO				UV/Vis, ESI-MS
2008 [76]	shellfish purple and precursors (12)	DMF	Phenomenex Synergi Hydro-RP, C18; 250×4.6; 4	A: water/0.1% form. acid B: ACN	18; 0.3	UV/Vis; ESI-MS
2008 [93]	flavonoids, anthraquinoids, indigoids, tannins, orchill	DMF, 140 ºC; water/MeOH/37% HCl 1:1:2, 100 ºC, rediss. DMF	Phenomenex Luna C18, 100×2	A: water B: MeOH 10% C: 5% ph. acid	27	UV/Vis

Table 2.1 Continued

2009 [38]	indigo, flavonoids, coumarins (12)	MeOH/acetone/ water/ox. acid 0.2 M 3:3:4: 0.1, 60 ºC; DMF, 60 ºC; rediss. water/MeOH 1:1	Nucleosil RP-18; 250×4.6; 5; 35 ºC	A: water/0.3% perchloric acid B: MeOH	40; 1.7	UV/Vis;
			Polaris C18-A; 150×2; 5; 35 ºC	A: water/0.8% form. acid B: ACN	40; 0.3	UV/Vis; ESI-MS
2010 [58]	anthraquinoids, flavonoids, indigotin, tannins	water/MeOH/37% HCl 1:1:2, 100 ºC, MeOH/DMF 1:1, 100 ºC form. acid/MeOH 5:95, 50 ºC, MeOH/DMF 1:1, 100 ºC	Phenomenex Luna, C18; 150×2.1; 5; 35ºC	A: water/0.1% TFA B: ACN	50; 0.5	UV/Vis
2010 [69]	anthraquinoids, tannins (5)	water/MeOH/37% HCl 1:1:2, 100 ºC; rediss. water/MeOH 1:2	Nova-Pack C18; 150×3.9; 4; 30 ºC	A: water/0.1% TFA B: ACN/0.1% TFA	45; 0.5	UV/Vis
2011 [70] 2012 [71]	flavonoids, tannins(4) (8)					
2011 [83]	shellfish purple (3)	DMF, 115 ºC	Phenomenex Luna, C18; 150×4.6; 3	A: water B: ACN	10; 0.9	UV/Vis; APPI-MS, SIM
2012 [75]	shellfish purple (7)	DMF, 110 ºC				
2011 [48]	anthraquinoids (insect reds) (6)	form. acid/MeOH 5:95, 60 ºC; rediss. water/MeOH/0.3% perchloric acid 50:20:30 water/MeOH/37% HCl 1:1:2, 60 ºC; rediss. water/MeOH/ 0.3% perchloric acid 50:20:30 0.2 M ox. acid/acetone/MeOH/water 0.1:3:3:4, 60 ºC; rediss. water/MeOH/0.3% perchloric acid 50:20:30 2 M TFA, 60 ºC; rediss. water/MeOH/0.3% perchloric acid 50:20:30	Zorbax Eclipse Plus, C18; 150×2.1; 5; 35 ºC	A: water/0.3% perchloric acid B: MeOH	30; 0.5	UV/Vis; ESI-MS
2011 [3]	shellfish purple (7)	DMF, 60 ºC	Wakosil II 5C18RS, C18; 250×4.6; 5; 40 ºC	A: water/0.1% TFA B :ACN/0.1% TFA	21[b]; 0.4	UV/Vis
2011 [42]	anthraquinoids, flavonoids, indigo, tannins, carthamin (18)	DMSO, 60 ºC; HCl/MeOH 1:30, 60 ºC	Wakosil II 5C18RS; 250×4.6; 5	A: water/0.1% TFA B: ACN/0.1% TFA	60; 0.8-1	UV/Vis, 10-70 ng/ml
2011 [89]	shellfish purple (14)	DMSO, 30 ºC, 70 ºC	Alltima[c], C18; 150×2.1; 3; 70 ºC	A: water B:ACN 10% C: 1% MSA	26[b]; 0.3	UV/Vis
2012 [44]	anthraquinoids, flavonoids, indigotin (28)	water/MeOH/37% HCl 1:1:2, 100 ºC, rediss. water/MeOH 1:1; DMF, 140 ºC	LiChrosorb-C18; 125×4; 5	A: MeOH B: MeOH/water 1:9 10% C: 5% ph. acid	35; 1.2	UV/Vis
2011 [51] 2012 [44]		water/MeOH/37% HCl 1:1:2, 105 ºC, rediss. water/MeOH 1:1	Zorbax C18; 150×4.6; 5; 40 ºC	A: water/0.2% form. acid B: MeOH/ACN 1:1	18; 0.8	UV/Vis; ESI-MS, SIM, MRM
2010 [43]	(14)					

Table 2.1 Continued

2011 [73]	anthraquinoids (30)	MeOH/ACN/4 M HF 1:1:2, 45 ºC; rediss. DMSO	Interchim Uptisphere NEC[c], C18; 150×2.0; 5; 30 ºC	A: water B: ACN 10% C: 1% form. acid	86; 0.2	UV/Vis
			Hypersil Gold[c], C18; 150×4.6; 5; 30 ºC			
2011 [74]			Synaptec Caltrex Resorcinaren[c] Calixarene Resorcinol; 250×4; 5; 30 ºC		91; 1.0	
			Pursuit XRs DP[c], Diphenyl; 250×4.6; 5; 30ºC			
			Luna Phenyl-Hexyl[c]; 250×2; 5; 30 ºC		86; 0.2	
2011 [40]	anthraquinoids, flavonoids, indigo, polyenes (24)	water/MeOH/37% HCl 1:1:2, 100 ºC; all rediss. water/MeOH 1:1	Phenomenex Luna C18(2); 100×2.0; 3; 25 ºC	A: water/10% MeOH B: MeOH 10% C: 2% form. acid	50; 0.160	UV/Vis; MS
		MeOH/form. acid 95:5, 40 ºC; water/MeOH/37% HCl 1:1:2, 100 ºC				
		MeOH/acetone/ water/0.21 M ox. acid 30:30:40:1, 60 ºC; water/MeOH/37% HCl 1:1:2, 100 ºC				
		MeOH/acetone/water/HF 30:30:40:1; water/MeOH/37% HCl 1:1:2, 100 ºC				
2011 [41]	anthraquinoids, flavonoids and glycosides, indigo, brazilwood, logwood (11)	water/MeOH/37% HCl 1:1:2, 100 ºC	Lichrocart Purospher Star RP-18; 250×4.6; 5; 30 ºC	A: water/2.5 % ACN/0.5 % form. acid B: ACN	15; 1.0	UV/Vis
		water/MeOH/37% HCl 1:1:2, 100 ºC, MeOH/DMF1:1, 100 ºC				
		form. acid/MeOH 1:19, 40 ºC				
		0.001 M Na_2EDTA/ACN/MeOH 1:5:44, 60ºC				
		0.1% Na_2EDTA/water/DMF 1:1, 100 ºC	Fortis C18; 150×2.1; 3; 30 ºC	A: water/0.1 % form. acid B: ACN	30	UV/Vis ESI-MS
		2 M ox. acid/acetone/MeOH/water 1:30:30:40, 60 ºC				
		pyridine, 100 ºC				
		DMF, 100 ºC				
2012 [87]	shellfish purple (12)	DMSO	Hypersil Gold C18; 50×2.1; 1.9; 40 ºC	A: water/0.1% form. acid B: ACN/0.1% form. acid	24[b]; 0.3	UV/Vis, HESI-MS
2013 [84]	flavonoids, anthraquinoids, indigotin, curcumin (9)	DMF; DMF 90 ºC	UHPLC BEH Shield RP18[c]; 150×2.1; 1.7; 40ºC	A: water/10% MeOH B: MeOH C: water/1% form. acid	40; 0.2	UV/Vis, 0.046-0.303 ng (except curcumin)
			HPLC Phenomenex Luna C18; 150×2; 3; 35 ºC	C: 5% ph. acid		0.140-0.321 ng (except curcumin)
2012 [56]	indigotin, indirubin (2)	6 M HCl/MeOH 50:50, 95 ºC; rediss. MeOH:DMF 1:1	Zorbax Extend-C18 RRHT; 50×2.1; 1.8; 35ºC	A: water/0.1% form. acid B: ACN	25; 0.8	UV/Vis; ESI with superheated N_2

Table 2.1 Continued

2014 [72]	sawwort flavonoids (18)	MeOH/form. acid 9:1, 60 ºC; MeOH/37% HCl 7:1, 60 ºC	Zorbax SB-Phenyl; 150×4.6; 3.5	A: water/0.15% form. acid B: MeOH	30; 0.5	UV/Vis; ESI
2012 [64]	flavonoids, anthraquinoids, (8)	water/MeOH 1:1, 2 M HCl water/MeOH 1:1, 2 M EDTA water/MeOH 1:1, 2 M TFA	Acquity UPLC BEH C18[c]; 100×2.1; 1.7; 30ºC	A: water/0.1% form. acid B: ACN/0.1% form. acid	6; 0.25	UV/Vis
	indigotine	DMF, 100 ºC THF				
2013 [65]	insect anthraquinoids (7)	water/MeOH/37% HCl 1:1:2, 100 ºC, rediss. DMSO	Alltima HP C18; 250×3; 5; 33 ºC	A: water/0.1% TFA B: ACN/0.1% TFA	35; 0.5	UV/Vis
				A: water/amm. form. buffer, pH 3 B: ACN		ESI, APCI
2013 [53]	shellfish purple (4)	DMSO, 150 ºC	Symmetry C18; 150×3; 5	A: water B: MeOH	30; 0.6	UV/Vis
2012 [52]	anthraquinoids (12)	water/MeOH/37% HCl 1:1:2, 105 ºC, rediss. water/MeOH 1:1	Zorbax C18; 150×4.6, 5; 40 ºC	A: water/0.2% form. acid B: MeOH/ACN 1:1	20; 0.8	UV/Vis, ESI
2014 [94]	flavonols (13)	pyridine/water/1 M ox. acid 95:95:10, 100 ºC	Vydac C18; 250×2.1; 5	A: water/0.1% form. acid B: ACN/0.1% form. acid	35	UV/Vis, ESI
2013 [10]	anthraquinoids, flavonoids, indigotin, brazilein (10)	water/MeOH/37% HCl 1:1:2, 100 ºC; all rediss. in water/MeOH water/MeOH/5 M form. acid 1:1:2, 0.5 µM EDTA, 100 ºC DMF, 60 ºC	Synergi C18; 150×2.1; 3	A: water/0.15% form. acid B: ACN/0.15% form. acid	30; 0.2	UV/Vis, ESI

[a] a number in brackets indicates the number of resolved or identified compounds (e.g. coeluting compounds can be distinguished by MS)

[b] the last eluting compound

[c] the most efficient columns within investigated in the study

* references in this table are related to the cited work [10]

2.2.2 Identification of insoluble blue pigments

Indigo and Prussian blue (PB) belong to important blue colorants and their distinguishing is useful for dating of artworks. Indigo has been produced from plant material (mainly from woad, *Isatis tinctoria* L. and indigo-plant, *Indigofera tinctoria* L.) since ancient times and was synthesized at the end of the 19th century. Due to its high lightfastness it was used not only as a textile dye but as a pigment in medieval paintings, illuminations, sculptures and frescos as well [13,43]. The coloring matter of indigo is indigotine. It is not present in plants but derived from precursors (indoxyl glycosides: indican, isatan A, B, C) by subsequent water extraction, fermentation and oxidation [13]. When dealing with natural species, small amounts of indirubin, isoindirubin and isoindigo are formed [44] which could help to distinguish between the natural and synthetic dye. PB is a synthetic pigment (a ferric hexacyanoferrate (II) complex) obtained for the first time at the beginning of the 18th century in Berlin [45]. It has become used in watercolors and widely substituted indigo in oil paintings [46]. Discrimination between indigo and PB in oil paintings can be difficult since both pigments exhibit similar properties: dark-blue shade if not mixed with other colorants, small-shaped particles, high tinting strength, insolubility in water and most common organic solvents [46].

A number of publications reflects the continuous interest in differentiation of PB and indigo. Several decades ago an interesting approach for identification of three major nineteenth-century blue colorants including Prussian blue and indigo was proposed [47]. After digestion with sulfuric acid and following separation into inorganic and organic phases PB was confirmed through wet chemical analysis for ferrocyanide ions and indigo was examined with UV/Vis and IR spectroscopy. The recent protocol for surface enhanced Raman spectrometry (SERS) identification of both colorants was based on similar sample treatment by sulfuric acid solubilizing PB and converting indigo to soluble indigo carmine [48]. LDI-MS, IR and Raman spectroscopy were compared for identification of three blue pigments (PB, indigo and copper phthalocyanine) in fresh and artificially aged samples. LDI-MS detected the lowest content of pigments with one exception – PB in the aged sample. For comparison, indigo was found even at concentration of 0.01% wt, whilst LOD of PB was 0.3% wt of pigment in the mixture [49]. Other MS methods were reported but not for simultaneous analysis of both pigments. PB was detected by mass spectrometry with UV LDI (λ = 337 nm) in pigments dispersed in water or linseed oil and applied to paper [50]. Indigo

was identified in modeled egg tempera by atmospheric pressure MALDI [51]. Therefore, the issue of simple and fast method for identification of these both pigments even if present in mixtures remains relevant.

Chapter 5.1 of this thesis describes a simple and effective protocol based on conversion/solubilization of pigments and flow injection analysis (FIA) with ESI-MS detection (**Fig. 5.1**). PB or indigo was identified in samples of the 20th century paintings. The new procedure has helped with evaluation of the historical painting 'Crucifixion' from St. Šebestián church in Mikulov [9].

2.3 Differentiation of plant gum binders by SFC/MS

2.3.1 Applications of plant gums

Plant-derived gums have been used as adhesives, binders, thickening, gelling, emulsifying and stabilizing agents for many ages. Traces of a plant gum were found in Egyptian mummies dated as early as 5th millennium BC [52]. The most well-known representative is gum Arabic derived from *Acacia* trees due to its practical and industrial importance. Other widely used binders include fruit tree gums: cherry (*Prunus avium*), peach (*Prunus persica*), plum (*Prunus* sp.), apricot (*Prunus* sp.) and some other exudates: tragacanth (*Astragalus* sp.), carob (*Ceratonia siliqua*), guar (*Cyanoposis tetragonolobus*), ghatti (*Anogeissus* sp.) and karaya (*Sterculia* sp.) [53].

For many centuries plant gums have been and still are used in various fields: food (bakery, beverages, confectionery, ice cream, flavor concentrates), medicinal products (solid medicines, cough syrups, vitamins, laxatives, dental fixtures, transdermal patches), cosmetics (perfumes, lotions, lubricants, creams, liquid soap), paper (envelopes, stamps, tobacco paper), textiles (sizing agents), lithography (protection of pigments), photography (gum dichromate photography), ceramics [53,54]. Manufacture of slurry explosives is another application of gums interesting from forensic viewpoint, e.g. guar and ghatti are used for thickening of nitrate salt solutions and improve resistance of explosive powders to water damage [53].

Since gums are water-soluble materials or at least swell rapidly in water forming colloids (tragacanth and karaya), they found extended application in water-based painting media, such as aquarelle, gouache, gum tempera as well as metallo-gallic inks [53,55]. Plant gums have been also used in dry painting media (pastels, pencils, charcoal). Incorporation of these binders in paints allows controlling of aggregation and wetting properties of pigments. Gum Arabic and tragacanth were applied in antiquity as binding media for pigments in Egyptian ointments used for mummification, in mural paintings in Christian catacombs, in paintings on silk and in manuscript illumination in the Middle Ages [53]. Additionally, plant gums have been used as components of varnishes (especially ghatti) and glues for furniture. Identification of a particular binder is useful for restoration purposes as well as for evaluation of object authenticity and its possible provenance. In this study we will focus on the three most widespread gums in artworks: gum Arabic, cherry gum and gum tragacanth.

2.3.2 Sources and composition of selected plant gums

Plant gums consist of branched polysaccharides with high molecular weight. Their exact structure remains unknown due to the high complexity. Their monosaccharide content is subject to variations depending on the biological source and processing. Gum exudates should be distinguished from plant resins which are based on terpene compounds. Most of the species produce exudate as a result of disease (gummosis) on their fruit and trunk, especially after mechanical injury followed by microbial or fungal attack [53,56]. Gums are formed by pathogenic degradation of certain cells or tissues. As a result of gum swelling, the bark may break and the gum exudes through the crack. The solution dries in contact with air and sunlight and forms hard, glasslike lumps of various colors, from transparent or white to dark brown, which can be easily collected. Gum formation protects an injured plant part by sealing the damaged region and eliminating infection and water loss. To increase yields for industrial gum production, intentional incisions in the bark are made or it can be stripped off a tree or shrub [53].

2.3.2.1 Gum Arabic

Gum Arabic produced from *Acacia senegal var. senegal* is considered as the premium quality (hashab), although other varieties, such as *A. Senegal var. karensis* or *A. seyal*, are commercially available [54]. These species naturally occur in tropical African regions and are also found in India. The major providers of gum Arabic are Sudan and Nigeria. The *Acacia senegal* gum is a branched, neutral or slightly acidic, complex polysaccharide obtained as a mixed calcium, magnesium, and potassium salt [57]. The main chain consists of 1,3-linked β-D-galactopyranosyl units. The side chains are composed of two to five 1,3-linked β-D-galactopyranosyl units, joined to the main chain by 1,6-linkages. Both the main and the side chains contain units of α-L-arabinofuranosyl, α-L-rhamnopyranosyl, β-D-glucuronopyranosyl, and 4-O-methyl-β-D-glucuronopyranosyl, the latter two mostly as end-units.

As was established by hydrophobic interaction and size exclusion chromatography with multi angle laser light scattering, refractive index (RI) and UV detectors, the major part of the *A. senegal var. senegal* gum (about 88% wt) consists of the so-called arabinogalactan (AG) fraction with weight-average molecular weight (M_w) of $1.5–4.0\times10^5$ [57,58]. The second major fraction (10% wt) is an arabinogalactan-protein complex (AGP) with M_w of $2.0–3.5\times10^6$ containing a greater proportion of protein (12% wt). The third minor fraction (<2% wt) with the highest protein content (47% wt) consists of several glycoproteins (GP) with M_w

of 3.0×10^5–2.7×10^6. The total M_w of this gum variety processed as a single peak is in the range 5–6.5×10^5. Gums collected from older trees (e.g. 15 years old) tend to have M_w shifted to higher values than younger ones (e.g. less than 10 years old). The total protein content is about 2%. The most abundant amino acids are hydroxyproline, serine and asparagine.

Gum produced from another variation of *A. senegal*, *var. karensis*, contains greater amount of AGP fraction (up to 35% wt) and has the total M_w around $1.0–1.2\times10^6$, almost double than that of *var. senegal* [58]. Gum derived from *A. seyal* contains the same fractions (AG, AGP, GP) composed of essentially the same monosaccharides and amino acids, however, distributed in different manner [59,60]. Protein content in *A. seyal* gum is lower than in *A. senegal var. senegal*, around 1% wt, and the total M_w is higher, in the range 5×10^5–1.1×10^6. According to the mentioned sources, *A. seyal* gum structure is denser than *A. senegal* and results in different rheological and emulsifying properties.

2.3.2.2 Cherry gum

Cherry gum is derived from the cultivated and wild trees of *Prunus avium* species widespread throughout Europe, Western Asia and North Africa. It has been collected and used in Europe for technical purposes for many centuries but was superseded by gum Arabic [53]. The gum is a by-product of the fruit cultivation and is usually collected during the off season. In the past, commercial cherry gum was usually contaminated with other fruit gums, such as plum and apricot. Gums from cultivated cherry trees dissolve in water completely whereas gums from wild trees dissolve only partially (14.5%), the residue swells and forms a jelly. The gum of the *P. avium* trees is an acidic polysaccharide with the total M_w of 1.6×10^5–2.5×10^5 [61,62]. It contains a core chain of β-D-galactopyranose units linked mostly by 1,6-bonds with glucuronic acid units attached in C-6 position. The side chains are formed by D-xylose, D-galactose, L-arabinose and, specifically for *var. duracina*, 4-O-methyl-D-glucuronic acid. The gum of the wild cherry tree, *P. avium* subsp. *avium*, contains an acidic polysaccharide formed by the β-D-galactopyranose units joined together predominantly by the 1,6-linkages [63]. The side chains are formed by β-D-glucuronic acid and units of L-arabinose, D-xylose, D-galactose. The total M_w of the polysaccharide is about 4.3×10^5. Both cultivated and wild gums contain D-mannose and traces of L-rhamnose with unknown exact linkage. The weight-average molecular mass and polydispersity of gum polysaccharides vary not only between species but from season to season as well [56]. A comparison of the gum exudates of several *P. avium* taxa showed quite large variations in their monosaccharide

content [53]. The variation in composition of the gums from the different varieties of *P. avium* is much greater than those found between gums from different varieties of the *Acacia* species.

2.3.2.3 Gum tragacanth

Gum tragacanth is produced by *Astragalus* species (mainly *A. gummifer*) growing at the Near East. The major producer of tragacanth gum is Iran. The gum is a complex, acidic proteoglycan, with the total M_w about 8×10^5 [53]. The main components are the soluble arabinogalactan part, tragacanthin, and an insoluble part called "bassorin" which swells to a gel-like state. The ratio of these components is variable (from 9:1 to 1:1). Traces of starch and cellulose are also reported. The soluble part tragacanthin contains two polysaccharide components: so-called tragacanthic acid, insoluble in ethanol, and arabinogalactan which dissolves in ethanol. Tragacanthic acid is formed by a linear backbone of 1,4-linked α-D-galacturonic acid carrying side-chains of β-D-xylopyranosyl, α-L-fucopyranosyl and β-D-galactopyranosyl residues attached through C-3 [64]. Arabinogalactan has a highly branched structure and contains 1,6- and 1,3-linked D-galactopyranose core with attached L-arabinofuranose, D-galacturonic acid and traces of L-rhamnose [64,65]. Bassorin has a structure of 1,4-linked α-D-galacturonopyranosyl chain substituted with β-D-xylopyranosyl, β-D-galactopyranosyl and α-L-fucosyl units [54]. Galacturonic acid in tragacanthin and bassorin is partially methoxylated and acetylated [66]. Carboxyl groups in galacturonic acid residues are present in their calcium, magnesium and potassium salt forms [53]. The total protein content is about 3-4% [67]. The major amino acids are hydroxyproline, aspartic acid, serine and histidine. Comparing to other gums, tragacanth solutions have higher viscosity at lower concentration and are stable over a wide pH range, down to pH 2 [53].

2.3.3 Analysis of plant gum-based binders

Traditional protocol for analysis of plant gum-based binders includes hydrolysis of binding material to simple monosaccharides and their separation following appropriate detection, although IR and Raman spectroscopy of intact binders are also utilized [53,68]. The number of sugars encountered in plant gums is relatively limited: L-arabinose (Ara), D-xylose (Xyl), L-fucose (Fuc), L-rhamnose (Rha), D-glucose (Glc), D-Galactose (Gal), D-Mannose (Man) and two sugar acids, D-glucuronic acid (GlcA) and D-galacturonic acid (GalA). Classification is based mainly on qualitative information (decisional scheme, see example in **Fig. 2.1**) since plant gums contain monosaccharides specific to their natural sources [69-71].

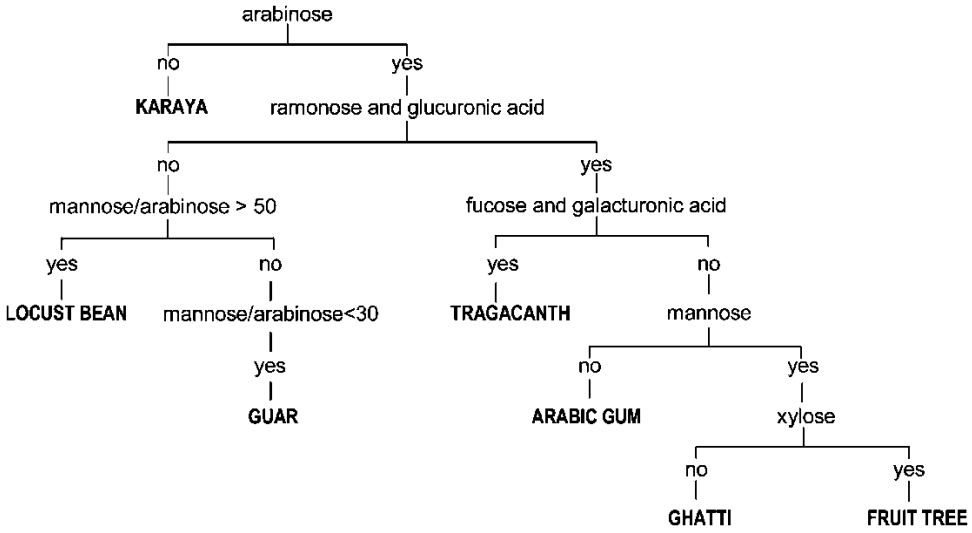

Figure 2.1 Decisional scheme for the identification of polysaccharide gums, adopted from [69].

For instance, Fuc is found only in tragacanth, so it serves as a specific marker for this gum. Direct quantitation is often complicated due to a very small amount of available sample (especially regarding art objects) and unknown content of a binder in the paint layer. Additionally, the actual composition of an art or historical sample might be affected by different factors: presence of other saccharide materials, biological attack and effect of aging [71,72]. For example, Xyl and Man cannot be considered as markers for fruit tree gums as they might derive from softwood. Egg binder interferes with Man identification. Rha and uronic acids are subjected to degradation in the presence of certain pigments. Therefore, other methods must be used for their classification. Relative ratio of monosaccharides [73], principal component analysis (PCA) [74,75] and cluster analysis [75] were applied for discrimination between plant gums.

2.3.3.2 Hydrolysis of plant gums

On the one hand, plant gums require hydrolysis under mild conditions to avoid possible degradation of monosaccharides, especially labile ones. On the other hand, prolonged treatment (up to 24 hours) with heated strong acid (HCl, H_2SO_4, TFA) is needed for complete hydrolysis of glycosidic bonds [53]. Strong mineral acids are not compatible with MS detection and must be removed prior analysis. Methanolysis and thermally assisted alkaline hydrolysis with methylation were used in combination with GC/MS [53,73,75]. Complete monosaccharide profile could not be obtained in those cases. TFA was successfully used in microwave-assisted hydrolysis (MAH) [74]. Comparing to the traditional sample treatment,

MAH is more efficient since molecules absorb microwave energy directly and hydrolyze much faster. To use all benefits of MAH special laboratory ovens and pressure-resistant vessels are required. Samples can be treated at higher temperature (100–120 ºC) without boiling, resulting in higher monosaccharide yield and avoiding sample loss. However, a conventional domestic microwave oven was used for complete hydrolysis of guar seed gum in 2 minutes [76]. Since the initial goal of our work was proving applicability of SFC/MS for analysis of monosaccharides, we only verified theoretical suitability of MAH. Therefore, we used a similar domestic set-up ensuring additional safety measures (see section **4.2, Sample preparation**).

2.3.3.3 Detection of monosaccharides

Conventional HPLC, CE and SFC detectors, such as UV/Vis, are not effective for monosaccharides since they are lacking a chromophore group. Refractive index (RI) and evaporative light scattering detectors (ELSD) are traditionally used [77]. Their disadvantage is low selectivity and low sensitivity. Pulsed amperometric detection in combination with ion-exchange chromatography was reported [53,74,78]. Occasionally, indirect UV/Vis or contactless conductivity detection was used in CE [53,70].

MS detection of monosaccharides also has flaws due to ionization issues, but in general it outperforms other types of detection. For instance, different sugar classes (pentoses, hexoses, deoxyhexoses, sugar acids) can be easily distinguished by MS. Of course, identification of individual representatives belonging to one class relying on MS only is very complicated or practically impossible (in case of mixture). Usually sugars are detected by ESI as Na^+ and NH_4^+ adducts in positive or as $[M-H]^-$ and $[M+CH_3COO]^-$ ions in negative mode [79-81].

2.3.3.4 Separation of monosaccharides

The earliest technique available for separation of monosaccharides was planar chromatography on paper and, later, TLC. The latter was still demanded in mid-90s when it was utilized for analysis of plant gums in art objects using silica gel plate with propan-1-ol/water/ammonia (79:20:1 v/v/v) system and p-anisidine/phthalic acid mixture as a detection reagent [82]. Although these techniques were fast and simple, they provided mainly qualitative information and consequently have been replaced by more sophisticated methods, such as LC and GC.

Conventional reversed phase HPLC is not suitable for underivatized monosaccharides because of their high polarity and thus, weak retention. Normal-phase and HILIC modes of chromatography are preferred for native sugars. Special stationary phases comprising amine, amide or cyclodextrin functionality are required [77,79,81]. For instance, Supelcosil NH_2 column with acetonitrile/water (80:20) as a mobile phase was used for characterization of neutral monosaccharides composition of *Acacia* exudate gums [60]. Alternatively, anion exchange chromatography can be utilized with minimal sample treatment [53,74,77,78]. GC can be used after prior derivatization of polar non-volatile compounds [53,73,75,83]. Usage of CE is complicated for neutral analytes and requires derivatization or highly alkaline medium to ionize sugars as well as high concentration of background electrolytes [53,70].

Applicability of SFC is limited by log *P* of target analytes (said to be in the range from -2 to 9, Waters recommendations). Therefore, this technique was never seriously considered as a method of choice for sugars (log *P* values below -2 [84]), although its range of application can be significantly expanded by use of appropriate modifiers and additives. Few papers on SFC of carbohydrates with ELSD detection were published in mid-90s and reviewed in ref. [85]. Separation of monosaccharides and sugar alcohols was studied on various polar phases. Among sugars of our interest, Rha, Xyl, Fru, Man and Glc were separated on a LiChrospher diol column with CO_2/methanol (84.5:15.5, v/v) mobile phase. Rha, Fru and Glc were distinguished on a RSil NO_2 column with CO_2/methanol (87:13, v/v). A LiChrosorb CN column allowed identification of Fru and Glc with CO_2/methanol gradient. Separation of pairs Ara-Xyl and Gal-Glc deduced from the retention times on two mentioned columns (diol and NO_2) was much worse. Zorbax Sil (bare silica) separated Rib, Rha, Fru, Man and Glc with CO_2/modifier (87:13, v/v), where modifier was a mixture of methanol/water/pyridine/triethylamine (91.95:4:4:0.05, v/v). The same polar Zorbax Sil and non-polar trimethylsilyl (TMS) phases were tested with methanol/water modifier (0, 4 and 8% of water) [86]. Water caused increase in retention of saccharides and anomer separation for Xyl and Gal. Based on the retention factors, separation of monosaccharides improved comparing to pure methanol, but pairs Ara-Xyl and Gal-Glc remained only partially resolved on both columns. Other saccharides useful for plant gum identification (Fuc, GlcA and GalA) were not included in any of the mentioned works on SFC.

Therefore, we aimed to push further the limits of modern SFC and investigate its applicability for analysis of all monosaccharides encountered in plant gum-based binders. For

the sake of comparison we included in our experiment sugars that are not present in plant gums but are widespread in nature and can be present in a sample as contaminants: D-ribose (Rib) and D-fructose (Fru).

2.4 Development of SFC/MS method for analysis of polar designer drugs

Part of materials shown in this section has been accepted for publication:

V. Pauk, V. Žihlová, L. Borovcová, V. Havlíček, K. Schug, K. Lemr, Fast Separation of Selected Cathinones and Phenylethylamines by Supercritical Fluid Chromatography, *J. Chromatogr. A* (2015).

2.4.1 New designer drugs

Number of reported synthetic drugs of abuse is increasing from year to year. Usually, a subtle change is made to an existing drug structure, such as replacement of a substituent or other innovation of molecule "design". Actually, new psychoactive substances are invented so fast that state authorities cannot take control over the situation in time and ban them (**Fig. 2.2**). In many countries new drugs are prohibited after they become a recognized concern. Thus, from the legislative point of view these compounds remain "legal" for some period and are distributed scot-free as souvenirs, collectibles or bath salts. To address the growing number of illegal designer stimulants in case of sports competitions, The World Anti-Doping Agency changed their policy of adding individual substances in the prohibited list to clearly identifying the whole family of phenylethylamine derivatives as being banned [87]. From alternative point of view, outlawing broad categories of substances on the state level could limit scientists to investigate their effects and to test them as potential medicines.

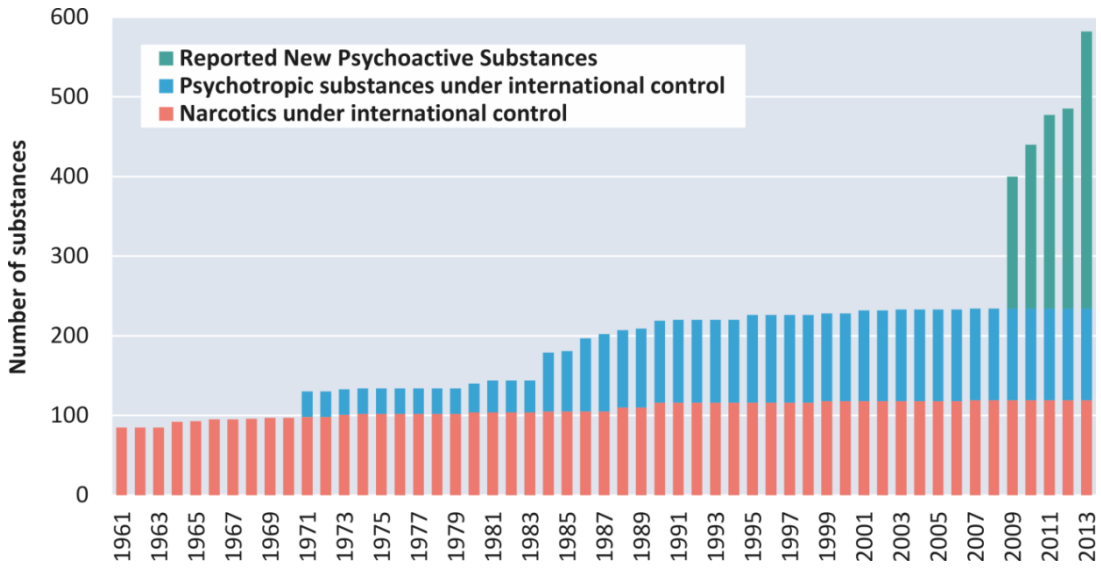

Figure 2.2 Number of new psychoactive substances not under international control and substances controlled under the international drug conventions, 1961–2013, adopted from [88].

According to the United Nations Office on Drugs and Crime (UNODC) report, synthetic cannabinoids, phenylethylamines and cathinones (substances possessing phenylethylamine core with carbonyl group attached to the β-carbon) are the most common constituents of "legal highs" [88]. The latter two classes of drugs together accounted for 42% of reports to UNODC in period from 2008 to 2013 (**Fig. 2.3**). There has been a 60-fold increase in the number of seizures of synthetic cathinones in Europe during this time [89]. 31 cathinones and 9 phenylethylamines were reported for the first time to the European Monitoring Centre for Drugs and Drug Addiction Early Warning System during the year 2014 alone [89].

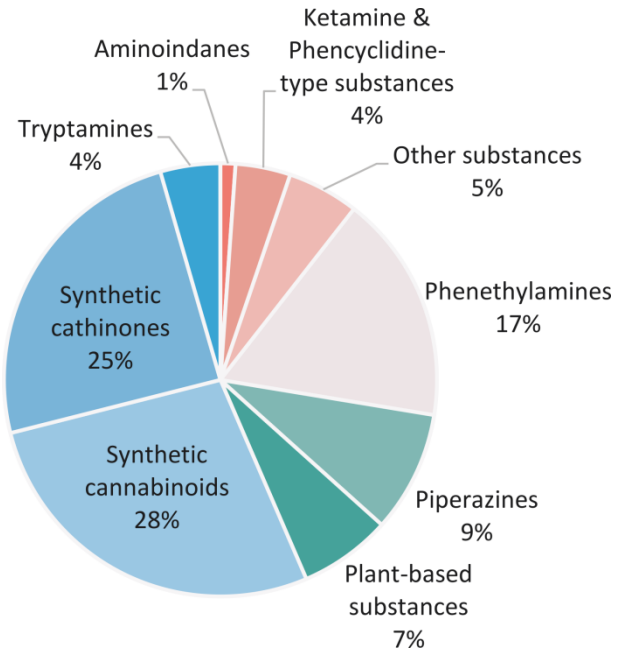

Figure 2.3 Total number of reports on new psychoactive substances to UNODC worldwide, 2008 to 2013, taken from [88].

While class of phenylethylamines has been known for a longer time and numerous representatives were synthesized and extensively described by Alexander Shulgin (mainly "2C" and "D" series) [90], very little was known about chemistry and biological activity of synthetic cathinones until recently. Their structure is based on a natural analogue – cathinone, the main psychoactive alkaloid of khat plant (*Catha edulis*) used in some countries of East Africa and Arabian Peninsula as a traditional social stimulant [91]. Over 20 million people chew fresh leaves of khat tree on a daily basis. Khat use has been reported among East African communities in Western Europe, USA and Australia. The effects observed following

khat consumption are similar to those of amphetamine stimulants. These are generally due to central nervous system stimulation and include euphoria, excitation, anorexia, increased respiration, hyperthermia, logorrhea, analgesia and increased sensory stimulation. Minor psychotoxic reactions (insomnia, anxiety, dizziness, irritability and aggression) usually occur after moderate intake of khat, however, serious psychiatric disorders are associated with long-term use.

Synthetic cathinones emerged on the recreational drug market relatively recently, in the second half of 2000s [92]. The most well-known are mephedrone, 3,4-methylenedioxypyrovalerone (MDPV), ethcathinone, butylone, flephedrone, 3-fluoromethcathinone, ethylone, methedrone, buphedrone, methylone and naphyrone to name a few. They are often sold in "head" shops and on websites as "bath salts", "plant feeders" or "research chemicals" in form of odorless, white, yellowish or brown powder or fine crystals, seldom as tablets or capsules. Although these products are often labeled with warnings "not for human use", they are intended to produce effects similar to that of illegal stimulants (e.g. MDMA, cocaine etc.). Similarly to phenylethylamines, cathinone derivatives can exist in two stereoisomeric forms which may differ in their potencies. The cathinone that occurs naturally in khat plant is the S-isomer. However, synthetic derivatives are very likely racemic mixtures. The most common administration routes are nasal and oral, although others were also reported. Cathinones are generally more hydrophilic than their phenylethylamine analogs and have lower values of blood-brain barrier permeation [93]. Therefore, higher doses are needed to produce similar effects. However, effective dosage and time of action may vary [92]. Thus, for mephedrone (4-methylmethcathinone, the most popular cathinone in Europe before it was banned) a typical nasal dosage is in the range 25–75 mg, effects appear within minutes and last less than an hour. Common oral dosages are between 150–250 mg, psychoactive effects appear after 45 min to 2 h and last for 2–4 h. On contrary to mephedrone, MDPV (the most prevalent cathinone in the USA) dosage starts from 3–5 mg up to 20 mg and effects can last over 6–8 h. The main mechanisms of cathinones action include inhibition of monoamine uptake transporters (dopamine transporter, noradrenaline transporter and serotonin transporter) and release of corresponding biogenic amines [92,94]. Cathinones undergo complex metabolism in human body which includes reduction of the keto group to a hydroxyl group, N-demethylation and oxidation of tolyl moiety to the corresponding alcohol or carboxylic acid. Subsequent metabolites are partially conjugated with glucuronides and

sulfates. In some cases significant part of the absorbed cathinones is excreted unchanged with urine [91,94]. Cathinones are often mixed with each other or with other drug classes (psychostimulants, β-blockers, anesthetics) which can lead to increased acute toxicity. Numerous fatal cases caused by cathinones intake were reported in the literature [92,94,95]. Since the trend of cathinones expansion will likely persist in the near future, development of new analytical methods for their identification is urgently required by analytical chemists, forensic experts, toxicologists, physicians and others.

2.4.2 Analysis of new designer drugs

Detection of drugs of abuse is usually performed in two major types of objects: drug samples itself (powders, crystals, pills etc.) and biological material. In some cases banknotes, clothing, fingerprints or even municipal wastewater are subjected to analysis of illegal drugs [8,95-98]. Although the same analytical method might be suitable for different objects (e.g. LC/MS) an appropriate sample preparation procedure should be selected for specific matrix. The biological materials commonly used in forensic toxicology of NDDs are urine, blood, and hair samples [91]. The choice of the biological matrix depends on the purpose of analysis. Analysis of urine and blood provides information on a recent or current exposure to a drug, while analysis of hair samples provides evidence of a long term and repeated use. There is a growing interest in the use of oral fluid as an alternative matrix for drug testing because of the significant advantages, such as a non-invasive collection under direct observation, absence of undue embarrassment or invasion of privacy, suitability for road-side and workplace testing and a good correlation with blood plasma analytical data [99,100].

Until recently, a two-step analytical strategy has been used by the forensic toxicology laboratories to monitor the abuse of illegal drugs [8,101]. The first step included preliminary high throughput screening method (color tests, immunoassay) aimed at identification of several classes of compounds (cannabinoids, cocaine, amphetamines, opiates, phencyclidine and sometimes barbiturates, benzodiazepines). The second, confirmation step was applied to samples identified as “presumptive positives” and based on a highly selective technique able to provide accurate recognition and quantitation of the target compounds (GC/MS).

Traditional forensic spot tests rely on the reaction of colorimetric reagents with nitrogen atom present in almost all NDDs (Marquis reagent – yellow to brown color with nitrogen-containing drugs, Simon’s reagent – blue to purple color with secondary amines, etc.) [102]. Zimmerman test was suggested for detection of cathinones since it relies on the presence of a

carbonyl group in close proximity to a methyl group on the same molecule and produces reddish-purple color [95]. Unfortunately, these reactions are lacking sensitivity. Selective ELISA (enzyme-linked immunosorbent assay) kits for controlled substances (e.g. amphetamine, methamphetamine, MDMA) have been developed and now are widely used as a screening step for detection of illegal drugs in urine or saliva [95,101,102]. However, they are ineffective towards NDDs because of weak cross-reactivity and, therefore, only a small part of NDDs can be detected [103]. By this time only assays for mephedrone/methcathinone, MDPV and three assays for synthetic cannabinoids are commercially available (Randox Toxicology, Crumlin, UK) [104].

More recently, the availability of high throughput techniques based on liquid chromatography coupled to triple quadrupole or to ion trap mass spectrometers prompted their incorporation into both steps of drug analysis strategy. Such chromatographic approach is by far the most used for screening and confirmation of analytes that cannot be detected by immunoassays [8,95,101,102]. GC and CE methods were also reported, although for a limited number of applications. It is worth noting that GC/MS has been the most widely applied technique for general unknown screening suitable to substances that are appropriate for GC and for EI or CI. However, while GC/MS necessitates sample clean-up, often extraction of the analytes and derivatization of polar substances (acylation, methylation), LC/MS makes possible direct injection of aqueous samples, such as diluted urine, and thus is suitable for a rapid, high throughput screening application (dilute and shoot strategy).

Bulk drugs do not require any sophisticated treatment, only homogenization, dissolution in an organic solvent and filtration. Common sample preparation methods for LC/MS analysis of NDDs in biological matrices are more complicated and usually include one of the following steps: deproteinization with methanol or acetonitrile; liquid-liquid extraction with organic solvents; solid-phase extraction on C18, C8 and/or cation-exchange sorbents [101,102]. Sometimes acid or enzymatic hydrolysis of metabolites is involved into the sample preparation for higher recovery of drugs conjugated with glucuronic acid or sulfate.

Separations are most frequently carried out in the reversed-phase mode on C18 stationary phases [101,102]. HILIC was reported as a possible alternative for improved resolution of synthetic cathinones [105]. Its acetonitrile-rich eluent favors desolvation in ESI source resulting in higher ionization efficiency and sensitivity (LODs at 0.02–0.5 ng/ml

level). The validated HILIC/MS method was utilized for detection, confirmation and quantitation of eleven designer cathinones in horse plasma for doping control at races.

SRM is the most sensitive and selective mode for the triple quadrupole and ion trap mass spectrometers. Using scheduled SRM survey followed by an information-dependent acquisition (enhanced product ion scan), multitarget screening LC/MS methods capable of detection and confirmation as many as 700–800 drugs, toxic compounds and their metabolites in biological specimens were developed [101]. The time needed for complete single sample acquisition and automated processing in these cases is around 20 minutes.

Among recent advantages in identification of completely new psychoactive substances high-resolution mass spectrometry (HRMS) must be highlighted. In addition to high-resolution instruments, such as FT-ICR and sector analyzers, high-end TOF and Orbitrap became available around 10 years ago. They combine versatility of LC and atmospheric pressure ionization with the specificity of high resolution and high accuracy mass measurements, robustness and high throughput [101,106]. Linked to a quadrupole with a collision cell or to a linear trap these machines allow full scan and MS/MS experiments, provide exact mass and hence, the elemental composition of parent as well as fragment ions. Structural hypothesis can thus be confirmed or deduced on the base of specific fragmentation pathways. LC/HRMS approaches have been successfully tested for untargeted screening of drugs and related compounds without the availability of primary reference standards, as is now the case with NDDs and most drug metabolites.

However, resolution of isomers remains an important issue in identification of NDDs. Among 125 cathinones ever reported by this time [107], 89 substances have at least one isomeric pair with absolutely the same monoisotopic mass. For instance, there are 15 isomers sharing the same stoichiometric formula $C_{12}H_{17}NO$. The analysis of three positional isomers of fluoromethcathinone (2-, 3- and 4-) revealed that their retention times under standard GC conditions were very similar, and slightly different after derivatization with acetic anhydride (difference in retention times was ~0.2 min), while the fragmentation patterns were almost identical [106]. LC/MS screening of methylated and fluorinated phenylethylamine and cathinone positional isomers with similar retention times and identical $[M+H]^+$ ions was also reported as challenging [108]. Whilst the 2-positional isomers were always distinguished, their respective 3- and 4-substituted analogues were poorly separated.

A whole bunch of alternative methods suitable for specific tasks of NDDs identification has been developed recently [8,95]. DART was utilized for rapid characterization of solid synthetic cathinones without sample pre-treatment. Ion mobility spectrometry with ESI and DART sources was also applied for screening of NDDs. Cyclic voltammetry with graphite electrodes demonstrated high potential for rapid and accurate quantitation of cathinones in seized street samples. Alternatively, Raman spectroscopy, especially SERS, can serve as a fast, non-destructive screening method for analysis of bulk drugs as well as trace evidence objects [95,98].

2.4.3 SFC of polar basic drugs

There is a constantly increasing interest to supercritical fluid chromatography (SFC) during last few years. This technique offers speed of analysis, high performance, reduced operation cost and environmental benefits. Modification of mobile phase with polar solvents and additives expands the range of potential analytes from various lipids and fat-soluble vitamins [109] to basic drugs [110], peptides and nucleobases [111]. So-called ultra-high performance supercritical fluid chromatography (UHPSFC) employing columns with sub-2 μm particles, combines advantages of both SFC and UHPLC and demonstrates high theoretical plate count and high linear mobile phase velocity. For example, more than 21000 plates were reached on a 100×3 mm, 1.7 μm column at 10.0 mm/s mobile phase velocity which is 4 times faster than optimum mobile phase velocity in UHPLC and 15 times faster than in conventional HPLC [112]. However, in order to utilize maximum performance of UHPSFC one must pay increased attention to the method development. On contrary to well-established procedures in UHPLC, guidelines and tutorials for relatively new UHPSFC are just being developed [113].

In the aforementioned context using of UHPSFC can be advantageous over other techniques especially in those areas where high-throughput separation is required, such as screening analysis of NDDs. These numerous and diverse synthetic drugs draw increasing attention in UHPLC screening methods [100,108,114-118]. Interestingly, recently published reviews highlight the importance of chromatography and mass spectrometry in analysis of NDDs but do not mention any applications of supercritical fluid chromatography [8,95,101]. Attempts to use SFC for separation of illegal drugs, particularly amphetamines, were described as early as in 1990 [119]. The drawback of the method was necessary derivatization of amino group due to its high polarity. In the later paper on stimulants, which included

amphetamine and methamphetamine, addition of 0.5% isopropylamine to methanol modifier was crucial for efficient separation [120]. Basic additive, 0.5% cyclohexylamine in isopropanol, was beneficial for chiral separation of amphetamine and methamphetamine enantiomers [121]. In the recent work, SFC behavior of basic compounds on a set of 2-ethylpyridine and hybrid silica stationary phases was investigated [110]. For compounds with pK_a values higher than 6-7 severe peak tailing occurred on 2-EP stationary phases due to secondary ionic interactions. The alternative solution, a hybrid silica BEH stationary phase with 20 mM ammonium hydroxide in methanol modifier, provided acceptable peak shapes for most of the investigated compounds and remained compatibility with MS. Amphetamine, MDA, MDEA and MDMA were included in the test set of hydrophilic drugs investigated on UHPSFC system, but no detailed chromatographic data is available on these compounds [122]. By this time no dedicated papers on SFC behavior of cathinones have been published. Taking into account increased polarity by introducing a carbonyl group, application of UHPSFC to analysis of cathinones appears even more challenging.

Therefore, purpose of this work was to test applicability of UHPSFC for analysis of cathinones and phenylethylamines with emphasis on resolution of isomeric compounds that cannot be unambiguously distinguished by MS even employing highly specific SRM mode. Further, the separation speed should be appropriate for fast screening of NDDs (less than five minutes). Revealing influences of the experimental variables on chromatographic behavior of selected cathinones and phenylethylamines helped us to find the most suitable conditions for fast and efficient separation. To the best knowledge of the author, this is the first work on UHPSFC of cathinones.

3 AIMS OF THE THESIS

The aim of this thesis was to develop new mass spectrometric methods for application in the field of forensic science. Particular tasks included:

- sensitive detection of historical pigments indigo and Prussian blue in paintings;
- analysis of natural polysaccharide-based binders in painting medium;
- identification of new designer drugs (cathinones and phenylethylamines) with emphasis on distinguishing isomeric substances.

The first and second tasks concern issues of authenticity, origin and dating of artworks and historical artifacts, while the third one deals with modern drugs of abuse. The main focus of this study was placed on sensitive detection, although simple and elegant sample preparation was also paid enough attention. Last but not the least, SFC separation conditions were investigated in details.

4 EXPERIMENTAL PART

4.1 Identification of Prussian blue and indigo by FIA/ESI-MS

Materials presented in this section have been published in ref. [9].

Chemicals and samples

Indigotin (95%) and sodium dithionite (approx. 85%) were purchased from Sigma-Aldrich (Prague, Czech Republic). Prussian blue, was synthesized from iron (III) chloride and potassium hexacyanoferrate (II) according to the literature [123]. Its purity was proved by ESI-MS (absence of soluble forms in filtrate obtained after washing of PB precipitate). Solutions were prepared using water purified by Direct-Q UV 3 (Millipore, Molsheim, France). Water for FIA experiments (LiChrosolv, HPLC grade) was purchased from Merck (Prague, Czech Republic), methanol (LC/MS grade) from Biosolve (Valkenswaard, The Netherlands). Other chemicals (Lachema, Brno, Czech Republic) were of analytical grade.

Samples of two oil paintings (beginning of the 20th century, 'Blue 1' and 'Blue 2' containing PB and indigo, respectively) and a blue microsample (scrape) from the painting 'Crucifixion', the St. Šebestián church on St. Hill in Mikulov, the Czech Republic, were kindly provided by IMAGO v.o.s. (Mikulov, Czech Republic).

Sample preparation

All solvents and solutions were degassed before the sample preparation using an ultrasonic bath (Elma S40H, Elmasonic, Singem, Germany). The ultrasonic bath was used in 'sweep' mode to disperse samples. A 50 ml volumetric flask with weighed pigment standard (5 mg) and 10 ml 0.2 M NaOH was filled with water, tightly covered with parafilm and placed in the ultrasonic bath for 30 min. PB decomposed, suspended indigotin was further reduced by addition of solid sodium dithionite (150 mg, sonicated for the next 30 min). Solutions were filtered (13 mm, 0.45 μm nylon LUT syringe filters, Cronus, Gloucester, UK) and diluted in 2 ml glass vials. The vials were first filled with appropriate volume of diluent and then a sample solution was added under the liquid surface. PB samples were diluted by water, indigotine – by 0.04 M NaOH with 3 mg/ml sodium dithionite. Blanks were prepared using the same procedure. The samples 'Blue 1' and 'Blue 2' (approx. 500 μg) were placed into a 10 ml volumetric flask, 2 ml 0.2 M NaOH and water were added. After 30 min of

sonication, 0.5 ml of suspension was filtered and collected into a 2 ml glass vial for identification of PB. Next, 30 mg sodium dithionite was added into the flask. The reaction mixture was closed with parafilm, sonicated for 30 min, filtered and used for indigo identification. A microsample of the painting 'Crucifixion' was treated directly in a vial due to its very small amount (unweightable, < 50 μg). Each standard sample was analyzed five times, blanks were analyzed ten times.

FIA/MS conditions

An Acquity UPLC system (Waters, Manchester, UK) was used for flow-injection experiments. Water/methanol (50:50, v/v) at flow rate 0.075 ml/min was used as a carrier liquid. The injection volume was 5 μl (10 μl sample loop). A PEEK capillary (0.25 mm I.D., 20 cm length, VICI AG, Schenkon, Switzerland) replaced a chromatographic column for FIA. A Q-TOF Premier mass spectrometer equipped with a Z-spray ESI source (Waters, Manchester, UK) monitored negative ions in full scan mode under the following settings:

capillary voltage:	2.3 kV;	desolvation gas temp.:	250 °C;
sampling cone:	30.0 V;	cone gas flow:	0 l/h;
extraction cone:	5.5 V;	mass range:	50 – 1000 m/z;
source temperature:	120 °C;	scan time:	1 s;
desolvation gas flow:	360 l/h;	inter-scan delay:	0.1 s;

in MS/MS mode:

mass range:	20 – 600 m/z;	collision voltage:	5 V, PB;
collision gas:	argon;	collision voltage:	25 V, leucoindigo.

For direct infusion experiments, a sample flow rate was set at 5 μl/min and desolvation gas at 150 l/h, other settings as above.

Data acquisition and processing were performed by MassLynx v. 4.0 software (Waters, Manchester, UK). Linear regression analysis was carried out using QC-Expert v. 3.2 software (TriloByte LTD, Pardubice, Czech Republic). LODs were determined according to ICH Q2(R1) (**Eq. 1**) [124].

$$LOD = 3.3\sigma/S \quad \textbf{Eq. 1}$$

σ – standard deviation of y-intercept;
S – slope of a calibration curve.

4.2 Differentiation of plant gum binders by SFC/MS

Chemicals

Carbon dioxide 4.8 grade (99.998%) was provided by SIAD (Prague, Czech Republic). Methanol LC/MS grade, formic acid (≥95%), acetic acid (≥99.7%), trifluoroacetic acid, TFA (≥99.0%) and ammonium hydroxide (≥25% ammonia in water) were purchased from Sigma-Aldrich (Prague, Czech Republic). Water LC/MS grade (Merck, Darmstadt, Germany) was used as mobile phase modifier in SFC. Water for sample preparation was purified by Milli-Q system (Millipore, Mollsheim, France). D-glucose, D-fructose, D-galactose, D-mannose, D-glucuronic acid, D-galacturonic acid monohydrate, L-rhamnose monohydrate, D-ribose, D-xylose and L-arabinose (all ≥97.0%) were purchased from Sigma-Aldrich (Prague, Czech Republic). L-fucose (≥97.0%) was supplied by Acros organics (Geel, Belgium). Structures of the investigated saccharides are shown in **Fig. 4.1**. Plant gums, Arabic (kibbled), cherry (kibbled), tragacanth (powder) were obtained from Kremer Pigmente (Aichstetten, Germany). Aquarelle half pans of fine grade Burnt Sienna, Sap Green and Ultramarine Blue (Pébéo, Gemenos, Cedex, France) were bought at a local art supplies shop in Olomouc. Samples of ceramic fragments with colored pattern on exterior surface supposedly dated to the 14th century were kindly provided by Martin Monik, Department of Geology, Faculty of Science, Palacký University.

Sample preparation

Gums (10 mg), aquarelle samples and paint from ceramic fragments (approx. 1 mg each) were sonicated for 10 minutes in aqueous 2 M TFA (5 ml) and hydrolyzed for approximately 4 minutes in 10 ml vials with punctured septa caps (to reduce excessive pressure) placed into a 250 ml beaker with 150 ml water (to absorb excessive microwave energy) in a domestic microwave oven (Sencor SMW 6023DS, 700 W). Microwave hydrolysis was immediately stopped after the beginning of boiling. Hydrolysates were filtered (13 mm, 0.22 μm nylon LUT syringe filters, Cronus, Gloucester, UK) and diluted 10 times with deionized water.

Instruments

An Acquity UPC^2 system (Waters, Manchester, UK) coupled to a Xevo TQD triple quadrupole mass spectrometer with a Z-spray electrospray source (Waters, Manchester, UK)

was used. Make-up liquid was delivered by a 515 HPLC Pump operated via a Pump Control Module II (both from Waters, Manchester, UK). Control of the instruments and data acquisition was performed using Waters MassLynx 4.1 software (Waters, Manchester, UK).

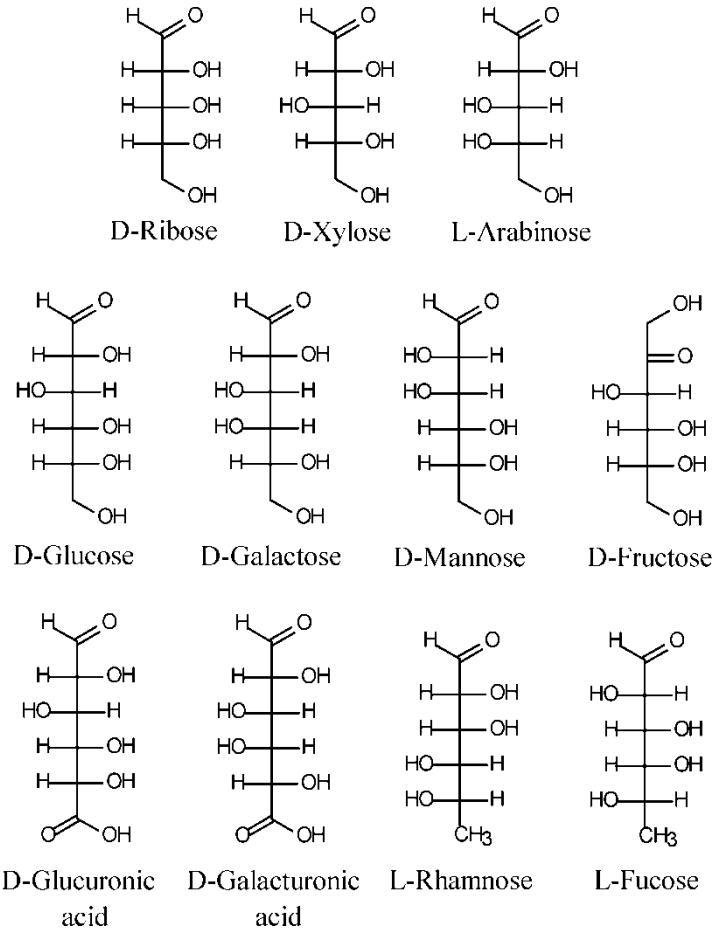

Figure 4.1 Structures of the investigated saccharides

MS detection

Source conditions were tuned during direct infusion of 2×10^{-5} M standard solutions (5 µl/min) with make-up liquid (0.400 ml/min) and set as follows for positive mode:

capillary voltage:	2.75 kV;	desolvation gas temp.:	250 °C;
source temperature:	150 °C;	cone gas flow:	30 l/h;
desolvation gas flow:	500 l/h;	extractor:	3.0 V.

for negative mode:

capillary voltage:	2.00 kV;	desolvation gas flow:	400 l/h;

other settings as above.

Continuous polarity switching was used during the method development and sample analysis.

Water/methanol (50:50, v/v) containing 10^{-6} M sodium acetate at flow rate 0.400 ml/min was used as a make-up liquid. SRM transitions were selected and tuned in manual mode by means of Waters IntelliStart software (Waters, Manchester, UK) and listed in **Tab. 4.1**. Losses of water and carbon dioxide were excluded due to the low selectivity.

Table 4.1 List of SRM transitions.

#	Substance	Molecular formula	MI mass	Precursor ion	Cone, V	SRM product ions[b)]	Collision voltage, V
1	galactose	$C_6H_{12}O_6$	180.06339	163.00	26	91.00 (Man)	10
2	fucose	$C_6H_{12}O_5$	164.06847	181.97	16	74.96	12
3	mannose	$C_6H_{12}O_6$	180.06339	198.00	20	127.00 (Fru, Gal)	16
4	pentoses	$C_5H_{10}O_5$	150.05283	323.00	15	173.00	10
5	deoxyhexoses	$C_6H_{12}O_5$	164.06847	350.91	20	186.99	10
6	hexoses	$C_6H_{12}O_6$	180.06339	382.97	22	202.99	10
7	uronic acids[a)]	$C_6H_{10}O_7$	194.04265	192.88	26	112.87 88.90 59.00	12 10 16

a) negative mode
b) interfering compounds

Stationary phases

Four Waters Acquity UPC^2 stationary phases were tested: **BEH** (silica) 1.7 µm, BEH **2-EP** (2-ethylpyridine) 1.7 µm, CSH **Fluoro-Phenyl** 1.7 µm and HSS **C18SB** 1.8 µm (**Fig. 4.2**). All columns possessed the same dimensions 100×3 mm i.d. and were used with in-line pre-column filters.

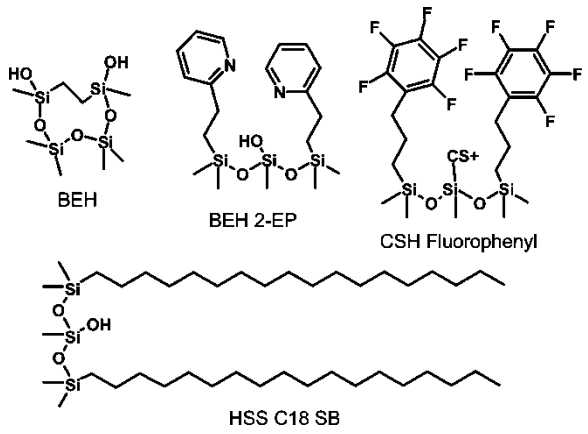

Figure 4.2 Chemistry of the tested stationary phases [125].

Chromatographic conditions and design of the experiment

Effect of several modifiers and additives including water, ethanol, acetonitrile, formic acid, acetic acid, TFA, ammonium hydroxide and ammonium formate was investigated. For each stationary phase two column temperatures (35 and 60 °C) were tested. Furthermore, 30 °C was tested on BEH and C18SB. Automatic backpressure regulator (ABPR) was set to 2000 psi (138 bar). Mobile phase flow rate was 2 ml/min. Elution program for column testing was set as follows: initial – 0% modifier; 8 min – 25% modifier; 9 to 10 min – 0% modifier.

Further, chromatographic conditions were evaluated separately for each column. Final conditions: ABPR pressure was 2000 psi (138 bar), column temperature was 35 ºC. Modifier consisted of methanol/water/formic acid (91:5:4 v/v/v). Mobile phase flow rate for the BEH column was 2.5 ml/min. Elution program for BEH: initial – 5% modifier; 9 min – 20% modifier; 10 to 11 – 5% modifier. Maximal pressure did not exceed 5300 psi (365 bar). Mobile phase flow rate for the C18SB column was 2.0 ml/min. Elution program for C18SB was set as follows: initial – 0% modifier; 5 min – 30% modifier; 6 to 7 min – 0% modifier. Maximal pressure did not exceed 5600 psi (386 bar).

Mixture (2 µl) containing all monosaccharides and uronic acids (2×10^{-5} M each) or a sample was injected. For method development blanks were run before each change of modifier or temperature for better column equilibration and controlling possible system contamination. For plant gums analysis blanks were run before each sample and samples were analyzed in triplicate. Chromatographic peaks were integrated manually using base-to-base method. Principal component analysis (PCA) was performed by means of OriginPro 2015 software (OriginLab Corporation, Northampton, MA, USA) using relative chromatographic peak areas of monosaccharides as variables.

4.3 Development of SFC/MS method for analysis of polar designer drugs

Chemicals

Carbon dioxide 4.8 grade (99.998%) was provided by SIAD (Prague, Czech Republic). Methanol LC/MS grade, formic acid (≥95%), acetic acid (≥99.7%) and ammonium hydroxide (≥25% ammonia in water) were purchased from Sigma-Aldrich (Prague, Czech Republic). Water was purified by Milli-Q system (Millipore, Mollsheim, France). 3-fluoromethcathinone (3-FMC) and 3-methylmethcathinone (3-MMC) hydrochlorides were obtained from Cayman Pharma (Neratovice, Czech Republic). All other investigated cathinones and phenylethylamines in form of hydrochlorides were purchased from Lipomed AG (Arlesheim, Switzerland). All investigated substances (**Fig. 4.3**) were of analytical grade purity. Working solutions (1.0 and 0.5 μg/ml) were prepared in methanol and kept in the autosampler at 10 °C.

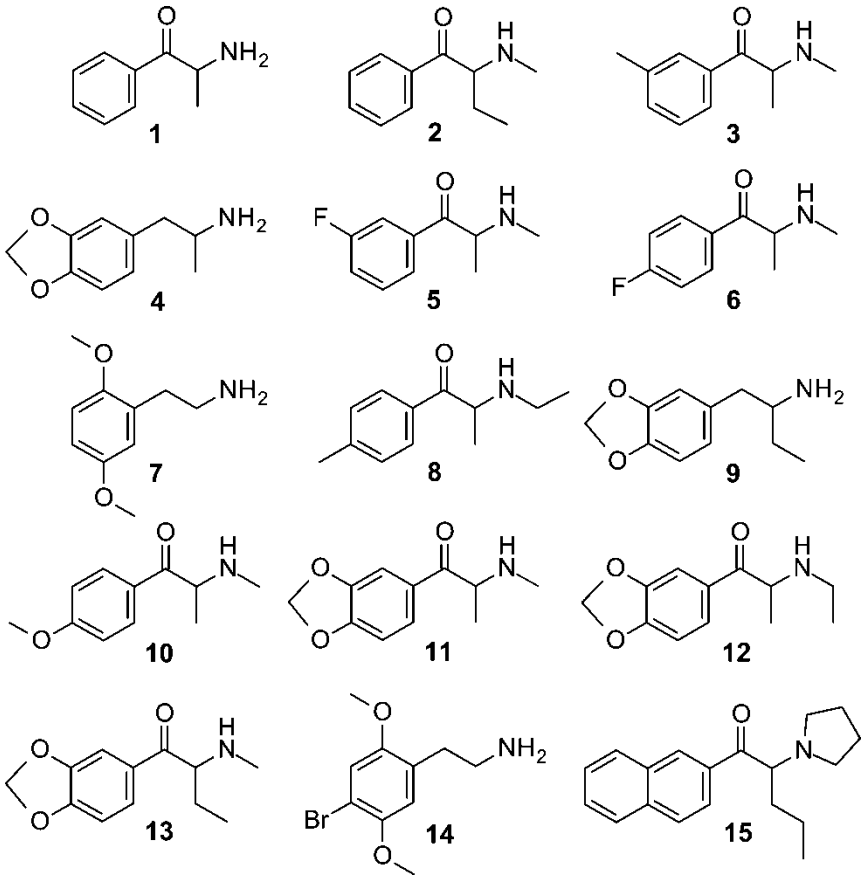

Figure 4.3 Structures of investigated cathinones and phenylethylamines: (**1**) cathinone; (**2**) buphedrone; (**3**) 3-MMC; (**4**) MDA; (**5**) 3-FMC; (**6**) flephedrone; (**7**) 2C-H; (**8**) 4-MEC; (**9**) BDB; (**10**) methedrone; (**11**) methylone; (**12**) ethylone; (**13**) butylone; (**14**) 2C-B; (**15**) naphyrone. Their properties are listed in Appendix C, **Tab. C1**.

Instruments

An Acquity UPC^2 system (Waters, Manchester, UK) coupled to a Xevo TQD triple quadrupole mass spectrometer with a Z-spray electrospray source (Waters, Manchester, UK) was used. Make-up liquid was delivered by a 515 HPLC Pump operated via a Pump Control Module II (both from Waters, Manchester, UK). Control of the instruments and data acquisition was performed using Waters MassLynx 4.1 software (Waters, Manchester, UK).

MS detection

Source conditions were tuned during combined infusion (direct infusion and make-up liquid) of 1 µg/ml solutions (buphedrone, 3-FMC, methedrone and ethylone) and set as follows:

capillary voltage:	2.80 kV;	desolvation gas temp.:	200 °C;
source temperature:	150 °C;	cone gas flow:	30 l/h;
desolvation gas flow:	500 l/h;	extractor:	3.0 V.

Methanol was used as a make-up liquid and delivered at 0.400 ml/min flow rate. SRM transitions (**Tab. 4.2**) were selected and collision energies tuned in automatic mode by means of Waters IntelliStart software (Waters, Manchester, UK) to get sufficient ion signal intensities. Losses of water and carbon dioxide were excluded due to low selectivity.

Stationary phases

Four Waters Acquity UPC^2 column chemistries were evaluated: **BEH** (silica) 1.7 µm, BEH **2-EP** (2-ethylpyridine) 1.7 µm, CSH **Fluoro-Phenyl** 1.7 µm and HSS **C18SB** 1.8 µm (**Fig. 4.2**). All columns possessed the same dimensions 100×3 mm i.d. and were used with in-line pre-column filters.

Chromatographic conditions and design of the experiment

All columns were tested under the same conditions to allow direct comparison of the results. Mobile phase flow rate and automatic backpressure regulator were set to default recommended values 2.0 ml/min and 2000 psi (138 bar), respectively. Six modifiers (pure methanol, 2% water in methanol, 20 mM ammonium hydroxide, 20 mM ammonium formate, 20 mM ammonium acetate and 20 mM formic acid in methanol) were utilized. Additionally, combination of 2% water and 20 mM ammonium formate in methanol was examined on HSS

C18SB and CSH Fluoro-Phenyl columns. Influence of additive concentration was investigated on BEH and Fluoro-Phenyl columns with 5, 10 and 20 mM ammonium hydroxide and ammonium formate in methanol. For each stationary phase three column temperatures (40, 50 and 60 °C) were tested. Furthermore, temperature 35 °C was tested on BEH. Gradient program was tuned to achieve the shortest runtime without compromising resolution of the early-eluting compounds and set as follows: initial – 6% modifier; 5 min – 30% modifier; 6 to 7 min – 6% of modifier. Mixture (2 µl) containing all 15 substances (500 ng/ml each) was injected. Blanks were run before each change of modifier or temperature for better column equilibration and controlling possible system contamination. Hold-up time was measured from diode array detector trace (195–300 nm) using nitrous oxide and corrected for mass spectrometer delay [126]. Peak width at half maximum ($W_{1/2}$) and asymmetry at 10% peak height were calculated using TargetLynx software (Waters, Manchester, UK). Peaks were considered Gaussian for asymmetry factor in the range 0.8 – 1.4. Resolution was calculated according to **Eq. 2**.

$$R = 1.176 \left(\frac{t_{\mathrm{RB}} - t_{\mathrm{RA}}}{W_{1/2\mathrm{A}} + W_{1/2\mathrm{B}}} \right) \qquad \textbf{Eq. 2}$$

t_{RA}, t_{RB} – retention times of compounds A and B, respectively;
$W_{1/2\mathrm{A}}, W_{1/2\mathrm{B}}$ – peak widths at half maximum of compounds A and B, respectively.

Table 4.2 List of investigated substances and their SRM transitions.

#	Substance	Molecular formula	MI mass	Precursor ion	Cone V	SRM product ions[a), b), c)]	Collision voltage, V
1	cathinone	$C_9H_{11}NO$	149.08406	149.92	30	**116.97** 76.98 (2C-H) 89.98	**18** 30 28
2	buphedrone	$C_{11}H_{15}NO$	177.11536	177.96	28	**130.96** (3-MMC) 90.96 (3-MMC) 147.02 (3-MMC) 76.98 (3-MMC)	**24** 20 12 34
3	3-MMC	$C_{11}H_{15}NO$	177.11536	177.96	30	**144.94** (buphedrone) 118.99 (buphedrone) 90.96 (buphedrone) 77.04 (buphedrone)	**20** 20 30 36
4	MDA	$C_{10}H_{13}NO_2$	179.09462	180.00	20	**104.96** 135.52 76.99	**22** 18 36
5	3-FMC	$C_{10}H_{12}NOF$	181.09028	181.93	32	148.98 (flephedrone) 102.93 (flephedrone, 2C-H) **122.96** (flephedrone) 76.98 (flephedrone, 2C-H)	18 26 **20** 32

Table 4.2 Continued

#	Substance	Molecular formula	MI mass	Precursor ion	Cone V	SRM product ions[a), b), c)]	Collision voltage, V
6	flephedrone	$C_{10}H_{12}NOF$	181.09028	181.93	32	**148.98** (3-FMC) 102.93 (3-FMC, 2C-H) 122.96 (3-FMC) 76.92 (3-FMC, 2C-H)	**20** 28 20 34
7	2C-H	$C_{10}H_{15}NO_2$	181.11027	181.95	14	**149.97** (2C-B) 134.95 (flephedrone, 3-FMC) 104.96 76.99 (flephedrone, 3-FMC)	**18** 24 22 36
8	4-MEC	$C_{12}H_{17}NO$	191.13101	191.97	30	**144.20** 118.98 90.95 130.94	**28** 24 34 24
9	BDB	$C_{11}H_{15}NO_2$	193.11027	194.01	24	**134.93** (methedrone) 76.98 (methedrone) 146.96 50.96	**14** 36 14 42
10	methedrone	$C_{11}H_{15}NO_2$	193.11027	194.01	28	**161.00** 57.99 145.92 (4-MEC) 134.99 (BDB)	**20** 12 28 20
11	methylone	$C_{11}H_{13}NO_3$	207.08953	207.93	18	**159.97** 132.00 57.99 90.96	**16** 24 14 34
12	ethylone	$C_{12}H_{15}NO_3$	221.10518	222.01	32	**174.12** (butylone) 146.03 (butylone) 90.95 72.03 (butylone)	**18** 28 38 14
13	butylone	$C_{12}H_{15}NO_3$	221.10518	222.01	32	**174.12** (ethylone) 145.96 (ethylone) 72.03 (ethylone) 190.96	**18** 24 16 12
14	2C-B	$C_{10}H_{14}NO_2Br$	259.02078 261.01873	259.86	20	**227.85** 90.95 105.91 163.99	**22** 40 42 20
15	naphyrone	$C_{19}H_{23}NO$	281.17796	282.08	40	**140.96** 211.00 126.19 155.00	**22** 18 30 30

a) product ions are listed in order of decreasing intensity;

b) ion in bold was used for SFC/MS detection;

c) interfering compounds are listed in brackets.

5 RESULTS AND DISCUSSION

5.1 Identification of Prussian blue and indigo by FIA/ESI-MS

Materials shown in this section have been published in the following articles:

V. Pauk, V. Havlíček, B. Papoušková, P. Sulovský, K. Lemr, Simultaneous identification of historical pigments Prussian blue and indigo in paintings by electrospray mass spectrometry, *J. Mass. Spectrom.* 48 (2013) 927–930 [9].

V. Pauk, P. Barták, K. Lemr, Characterization of natural organic colorants in historical and art objects by high-performance liquid chromatography, *J Sep Sci.* 37 (2014) 3393–410 [10].

5.1.1 Method development

PB (solubility product around 10^{-40}) and indigo are insoluble in water and common organic solvents [127,128]. Solvents like dimethyl sulfoxide (DMSO), dimethylformamide (DMF), tetrahydrofuran (THF) and concentrated HCl are often used for indigo extraction from paintings and plant materials, but they are incompatible with electrospray ionization [10]. To overcome low solubility, PB was quantitatively decomposed in alkaline solution to form iron (III) hydroxide and hexacyanoferrate (II) ions (**Fig. 5.1, a**) that can be detected by MS. Complete decomposition of PB was achieved only at pH higher than 11. Indigotin was reduced by sodium dithionite to leucoindigo at pH > 12 (**Fig. 5.1, b**). At higher pH values solubility of leucoindigo increases due to the formation of mono- and di-ionic forms [129,130]. Thus, eventual pH 12.3 was used. Higher pH was not tested because of the risk of instrument corrosion.

Sodium dithionite can be easily oxidized in solid state and in water solution by air oxygen [131]. Its concentration 1 mg/ml was sufficient to reduce indigotin to leucoindigo, but re-oxidation was observed during filtration. The excess of dithionite protected leucoindigo, but too high concentration (above 5 mg/ml) increased chemical noise in mass spectra and caused noticeable instrument contamination. Selected concentration 3 mg/ml allowed easy manipulation with samples in open vessels, filtration and storage in closed glass flasks under the laboratory conditions for at least one week without a significant decrease in ion intensity (less than 10%, Appendix A, **Fig. A1**).

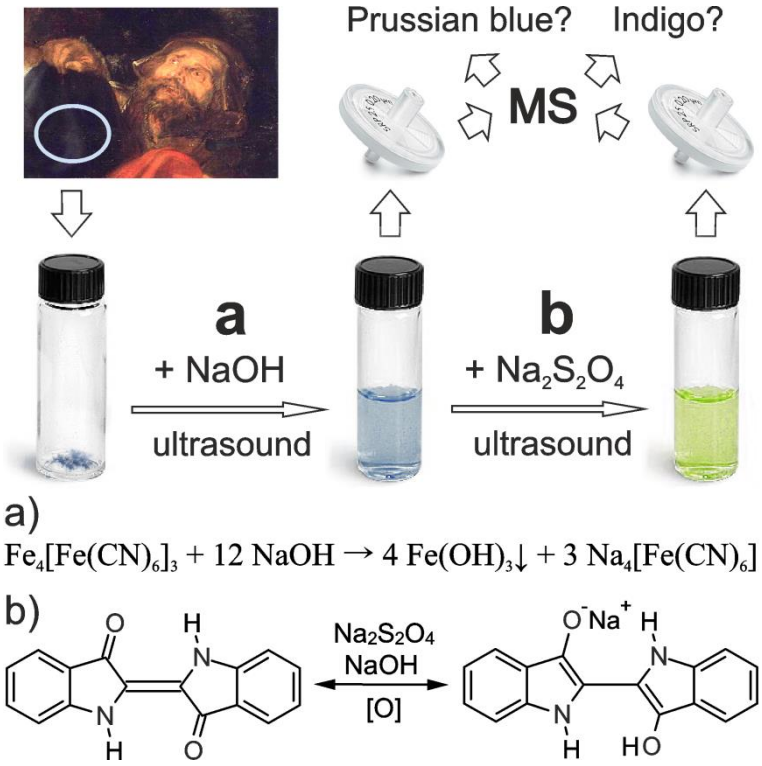

Figure 5.1 Protocol for analysis of paint microsamples: **a)** decomposition of PB; **b)** conversion of indigotin to soluble leucoindigo (adopted from [9]).

A spectrum of decomposed PB sample contained signals of $[Fe(CN)_3]^-$ at m/z 133.9445 (error 2.2 ppm), $[Fe(CN)_2]^-$ (m/z 107.9418; 7.5 ppm) and $[Fe(CN)_4]^-$ (m/z 159.9484, 6.9 ppm), in accordance with an ESI-MS spectrum of potassium hexacyanoferrate (II) (**Fig. 5.2, a**) and literature data [132]. Interestingly, potassium hexacyanoferrate (III) produced a very similar spectrum (data not shown). Besides accurate masses, a typical isotopic profile proved the composition of ions. Given m/z values correspond to the most abundant isotope ^{56}Fe.

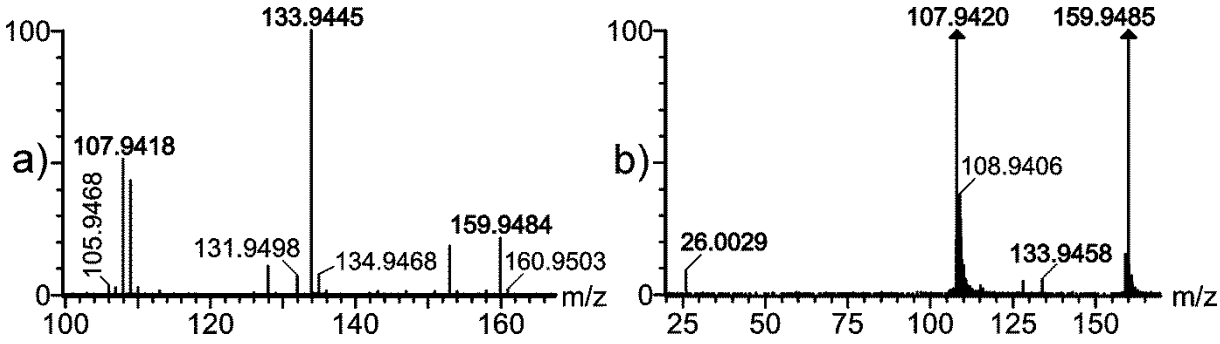

Figure 5.2 **a)** Mass spectrum of potassium hexacyanoferrate (II) 10 µg/ml in water; **b)** fragmentation spectrum of m/z 159.9485, see text for details.

Ferrous complexes with CN^- ligand were essential for PB identification. In general, only iron detection would not be sufficient as other ferrous pigments can be present, e.g. in multilayer paintings.

Ions at m/z 133.9445 and 107.9418 gave no intensive fragments. Starting from 10 eV, the signal of precursor ions significantly decreased and almost disappeared at 20 eV. It might be due to decomposition rendering CN^-, a little peak was found at m/z 26.0029 (error 7.7 ppm). Ion m/z 159.9485 easily fragmented to m/z 107.9420 (loss of $(CN)_2$), and disappeared at energies above 5 eV (**Fig. 5.2, b**).

Leucoindigo produced $[M\text{-}H]^-$ ion at m/z 263.0822 (error 0.4 ppm) (**Fig 5.3, a**). Its fragmentation rendered product ions useful for identification. The most abundant fragments were observed at m/z 245.0727 (4.9 ppm, $–H_2O$), 217.0770 (1.8 ppm, $–H_2O–CO$), 144.0444 (3.5 ppm, $–C_7H_5NO$) and 141.0446 (5.0 ppm, $–C_7H_6O_2$) (**Fig. 5.3, b**).

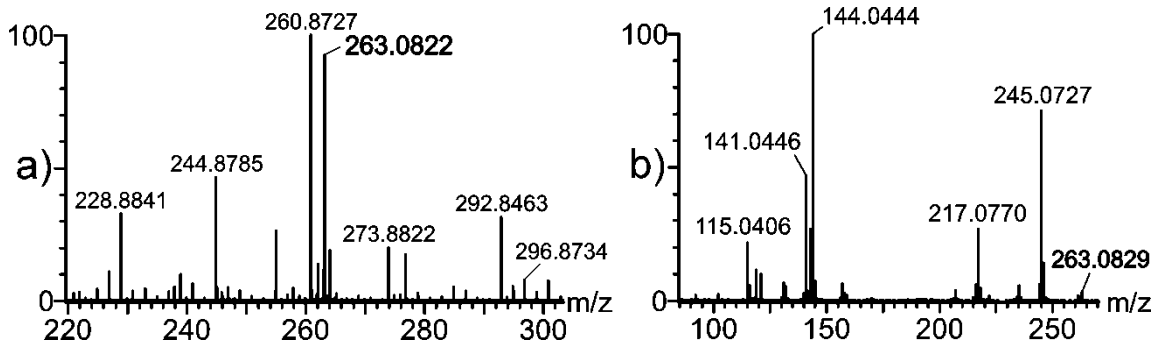

Figure 5.3 a) Mass spectrum of leucoindigo; **b)** fragmentation spectrum of leucoindigo.

Simultaneous analysis of both pigments within one spectrum was evaluated. After treatment with sodium dithionite an unidentified peak around m/z 133.946 interfered with PB signal in all samples and blank (Appendix A, **Fig. A2**). The problem was solved by the separate analysis of sample solution prior and after addition of sodium dithionite.

The automatic flow-injection analysis preserved samples from being re-oxidized by air oxygen (sealed sample vials) and provided short runtimes. Flow rates of 0.050, 0.075, 0.100, 0.125, 0.150 and 0.200 ml/min were tested. The largest peak area without peak tailing compromising detection was achieved at 0.075 ml/min with analysis time below two minutes (**Fig. 5.4**).

LODs and linearity were evaluated using 14 calibration solutions in the range 5–10000 ng/ml for both pigments (**Tab. 5.1**). Calibration curves for determination of LOD were constructed using seven points from 10 to 250 ng/ml or six points from 15 to 250 ng/ml for

PB or indigotin, respectively. The peaks reconstructed for m/z 133.946 or 263.082 using 0.01 Da window were integrated. Injected amount above 25 ng (concentration above 5000 ng/ml) worsened linearity but had no influence on pigment identification. For indigotin, the linearity range and LOD were comparable to an HPLC/MS method (30–4200 ng/ml, 50 pg on column) [133].

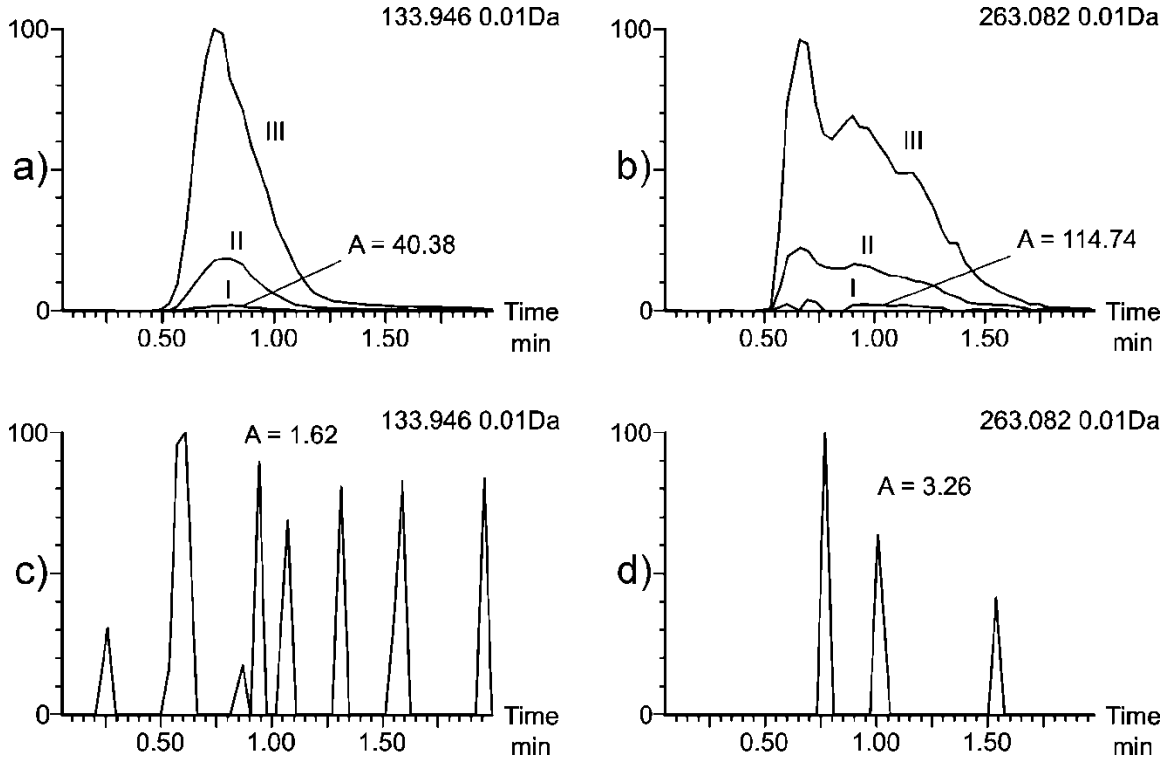

Figure 5.4 FIA mass-chronograms: **a)** PB, **b)** indigo. Curves I, II, III respond to 100, 1000 and 5000 ng/ml concentrations, respectively; **c)** and **d)** are corresponding blanks; integrated peak areas are shown.

Table 5.1 Regression parameters and LODs for PB and indigo.

Analyte	Range, ng/ml	Intercept		Slope		r	LOD, ng/ml		Absolute LOD, pg
		Value	SD	Value	SD		Value	CI	
Prussian blue	10 – 250	-4.8095	1.3001	0.4524	0.0110	0.9985	9.48	0.24	47
Leucoindigo	15 – 250	12.5383	3.3042	0.9294	0.0258	0.9985	11.72	0.34	59
Prussian blue	10 – 5000	-24.1591	11.5472	0.5338	0.0068	0.9992	-	-	-
Leucoindigo	15 – 5000	32.3216	25.8363	1.0331	0.0145	0.9991	-	-	-

5.1.2 Analysis of samples from oil paintings

LOD values indicated the feasibility of the developed protocol for real samples. It was further tested using two blue samples of oil paintings from the 20th century. 'Blue 1' was identified as PB (**Fig. 5.5, a**) with no detectable traces of indigo, 'Blue 2' contained indigo but not PB (**Fig. 5.5, b**), both in agreement with the known sample composition. It is worth noting, indigo can degrade in aged samples [134] but still unchanged can be identified in old painting [48].

Blue paint was sampled to examine the painting 'Crucifixion' from the St. Šebestián Church on Saint Hill in Mikulov, the Czech Republic. The painting is a palimpsest with an older layer different in arrangement of figures, allegedly dated to the 16th century. Electron probe microanalysis excluded blue pigments widely used from Middle Ages up to the 19th century like ultramarine, azurite, blue verditer, vivianite, smalt etc. Neither electron probe microanalysis nor Raman spectroscopy has rendered a clue for PB or indigo identification. Utilizing the developed protocol, no traces of indigo were found in the microsample of the painting 'Crucifixion', but the presence of PB was evident (**Fig. 5.5, c**). The result of analysis supports the hypothesis that the object was re-painted after the 18th century or later.

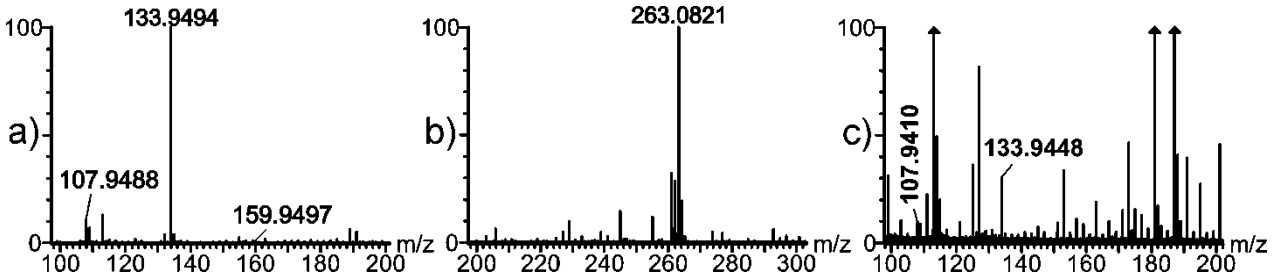

Figure 5.5 Analysis of three real samples of oil paintings: **a)** 'Blue 1' 20th century - PB detected; **b)**'Blue 2' 20th century - indigo detected; **c)** microsample of the painting 'Crucifixion' - PB detected.

5.1.3 Conclusion

The developed FIA/ESI-MS protocol proved to be successful in identification of pigments of high historical importance – PB and indigo. It offers indigotin LOD comparable to HPLC/MS. To our best knowledge, ESI-MS methods for simultaneous identification of PB and indigo were not described by the moment of this publication. The new method is rapid, simple and sufficiently sensitive and allows for mass spectrometric analysis without chromatographic separation. It represents a useful alternative to other methods applied in

simultaneous analysis of both pigments as LDI-MS, infrared or Raman spectroscopy and SERS [48,49]. Since absolute LOD were not explicitly given for the listed methods, the direct comparison with described FIA/ESI-MS has not been possible. The microsample of the painting ‘Crucifixion’ contained PB which could exclude Middle Ages origin of the painting. Since the painting is a palimpsest with cracks, analysis of other samples from different locations is suggested to confirm PB in both layers.

5.2 Differentiation of plant gum binders by SFC/MS

5.2.1 Method development

5.2.1.1 Mass spectrometric detection

The observed monosaccharide precursor ions were in agreement with the literature [79]. Generally, pentoses, hexoses and deoxyhexoses produced sodium adducts $[M+Na]^+$ and sodiated dimers $[2M+Na]^+$. $[M+Na]^+$ ions gave no fragmentation products except m/z 23 (Na^+) of low intensity. $[2M+Na]^+$ ions lost one monosaccharide moiety and $[M+Na]^+$ products were detected in MS/MS experiments. Therefore, sodiated dimers were chosen for SRM transitions (**Table 4.1**). Additionally, Gal in-source dehydration product m/z 163 $[M-H_2O+H]^+$ gave specific transition to m/z 91. Fuc, Man and Fru produced ammonium adducts $[M+NH_4]^+$ valuable for complementary confirmation. Signals of hexoses in negative mode $[M-H]^-$ and $[2M-H]^-$ were roughly 20 times weaker than $[2M+Na]^+$. Uronic acids formed $[M+Na]^+$ adducts in positive mode, but their fragmentation spectra were poor (only m/z 23). Thus, their deprotonated ions were used for SRM in negative mode. Finally, for detection of all substances in one chromatographic run continuous polarity switching was used.

Stable sodium adducts were observed without deliberate introduction of salts into the make-up liquid or mobile phase modifier. Exact sodium source remained unknown. Therefore, 10^{-6} M sodium acetate was added into the make-up liquid to prevent possible signal instability and reproducibility issues.

5.2.1.2 Additives and modifiers

Though for used mobile phase compositions rather subcritical conditions existed in separation system, supercritical fluid chromatography was used as a technical term throughout the text. Obviously, pure CO_2 was not suitable for separation of monosaccharides due to solubility issues and weak elution strength. Addition of the appropriate modifier was crucial for successful separation of such polar analytes. In general, monosaccharides and uronic acids showed very similar chromatographic behavior on all stationary phases under investigated conditions (Appendix B, **Fig. B1-B4**). Pure methanol used as modifier eluted all monosaccharides except the most polar uronic acids. Peaks were distorted and separation of individual hexoses, pentoses and deoxyhexoses was unacceptable. Addition of 2% water and increasing its concentration to 5% significantly improved chromatograms most probably due to masking of active sites on a stationary phase surface. Further increase of water content in

modifier (7-10%) caused pressure instability and system overpressure at higher percentage of modifier. It is not surprising, since supercritical temperature and pressure of water (647 K, 220 bar) are much higher than those of CO_2 (304 K, 74 bar) [135]. Most likely, mobile phase converted to a liquid state (one order of magnitude higher viscosity).

Mixture of methanol and water did not have enough strength to elute uronic acids. Additives such as 20 mM ammonium hydroxide and ammonium formate, alone and in combination with water, worsened peak shapes while formic acid produced sharper peaks. Mixture of 5% water and 2% HCOOH in methanol provided the best peak shapes and separation of individual monosaccharides. However, uronic acids were not completely eluted during the chromatographic run. Since their pK_a values are relatively low, 3.20 and 3.48 for glucuronic and galacturonic acid, respectively [136], strongly acidic pH around 1.2 is required for domination of non-ionized form. Increasing concentration of formic acid in modifier to 4% maintained separation of monosaccharides and allowed to elute uronic acids completely except from the 2-EP column, where strong electrostatic interaction between protonated stationary phase and remained fraction of dissociated acids likely occurred. This value might seem high, but at maximum point of modifier gradient (25%) content of formic acid in the mobile phase passing through column is only 1%.

Weaker CH_3COOH was not efficient in elution of uronic acids at used contents (2-4% in modifier), but separation of other monosaccharides was similar. 0.1% TFA with 5% water in methanol showed results comparable with 2% HCOOH concerning retention, peak shapes and separation, but signal of late-eluting compounds was significantly decreased, roughly 3 times for hexoses and 10 times for uronic acids.

Replacement of methanol with ethanol as a modifier in combination with 5% water and 2% formic acid rendered similar results, however several drawbacks were observed. Gal produced wide peaks (fronting and tailing) and peak heights of most sugars were lower (up to 50 % in some cases). Acetonitrile as modifier with 5% water and 2% formic acid failed to elute any of the studied substances.

5.2.1.3 Column temperature

Column temperature significantly affected interconversion of anomers. At 35 ºC several sugars provided two peaks corresponding to α and β anomers. Usually, separation of sugar anomers is seen as a drawback [86]. In our case, for some monosaccharides one of the anomers overlapped with another sugar but the second could be used for identification. At

60 ºC anomers merged into one peak and thus, identification of individual sugars became impossible (**Fig. 5.6**). Lower temperature (30 ºC) did not result in further improvement of separation on BEH and C18SB columns.

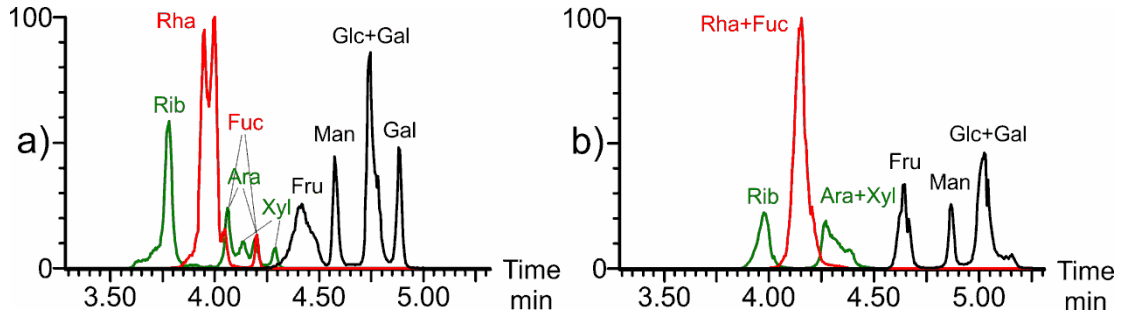

Figure 5.6 Separation of the standard mixture on C18SB, 5% H_2O, 2% HCOOH: **a)** 35 ºC; **b)** 60 ºC.

5.2.1.4 Stationary phases

Retention of monosaccharides on all phases strongly correlated with the number of hydroxyl groups. Thus, pentoses and deoxyhexoses were eluted first, then hexoses and, finally, uronic acids (**Fig. 5.7**). This elution order is in agreement with the literature [85] and strongly resembles a typical behavior of sugars in HILIC [77]. Uronic acids, however, were not separated and eluted as a broad band due to high polarity and presence of deprotonated form. SFC could not be a method of choice for quantitative analysis of these substances but at least their presence might be confirmed. Regarding separation, we observed three critical pairs common to all stationary phases: Fuc-Rha, Ara-Xyl and Gal-Glc.

Since neither stationary phase completely separated all monosaccharides, backpressure, mobile phase flow rate and gradient optimization was further considered separately for each column. Under the most suitable conditions BEH and C18SB clearly outperformed other stationary phases and allowed identification of each monosaccharide (**Fig. 5.8**). Due to a faster gradient peak height was roughly two times higher on the C18SB column than on BEH. The 2-EP column did not separate neither deoxyhexoses nor Xyl and Ara. As it was mentioned, uronic acids were not eluted from this column. The Fluoro-Phenyl column did not render separation of Xyl and Ara, deoxyhexoses were separated only partially.

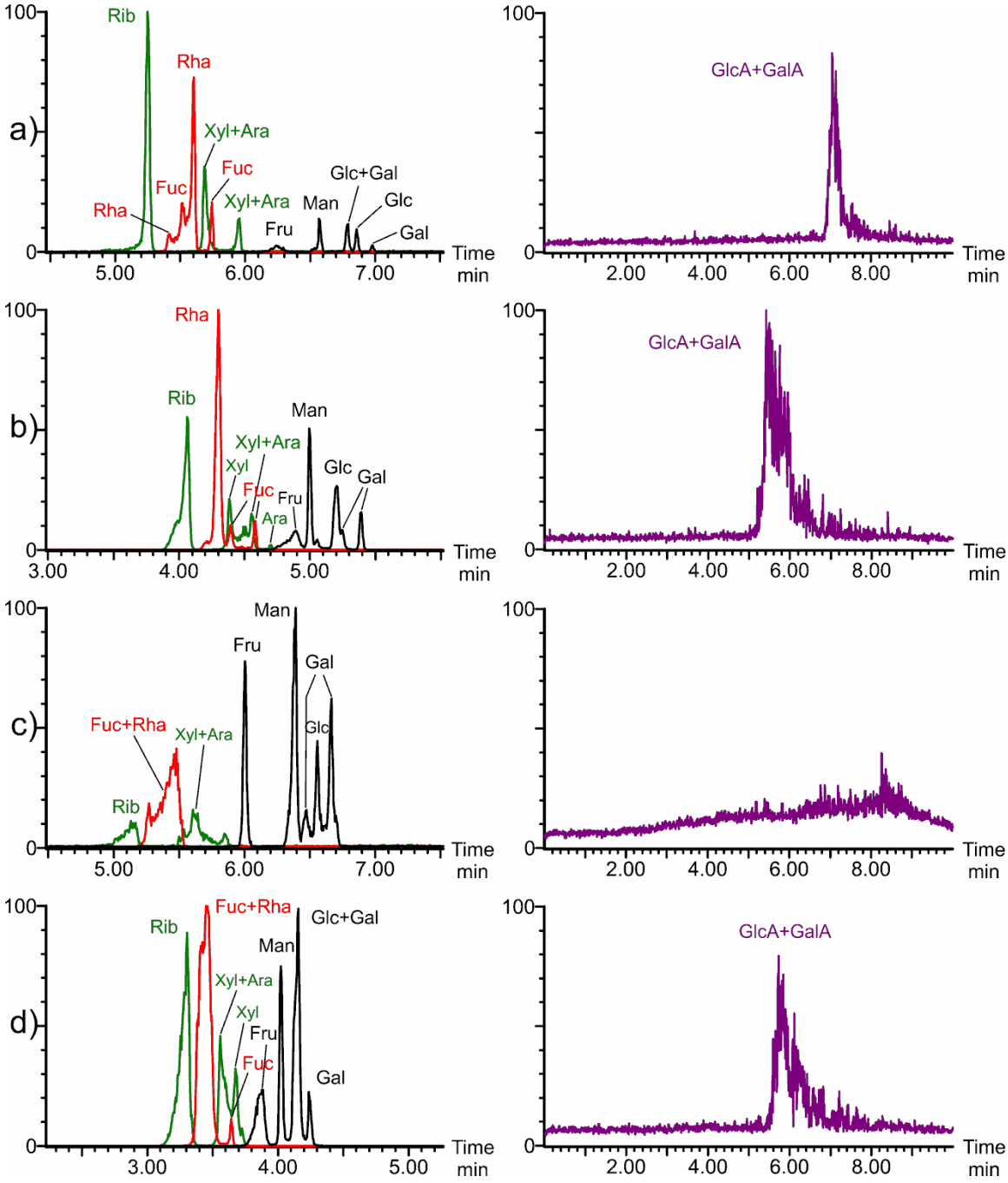

Figure 5.7 Separation of the standard mixture, 5% H_2O, 4% HCOOH in MeOH, 35 °C: **a)** BEH; **b)** C18SB; **c)** 2-EP; **d)** Fluoro-Phenyl. Left: positive MS mode, right: negative.

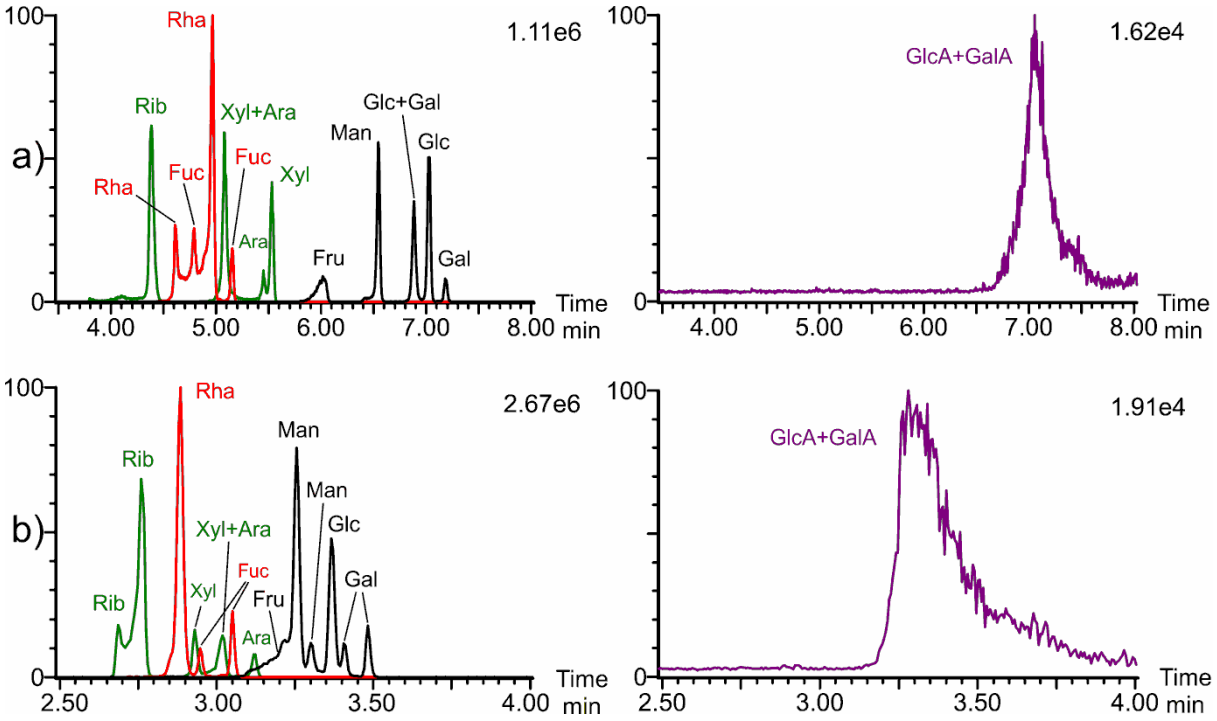

Figure 5.8 Optimized separation of the standard mixture, 35 °C: **a)** BEH, gradient: 5 to 20% modifier in 9 min, 2.5 ml/min; **b)** C18SB, gradient: 0 to 30% modifier in 5 min, 2.0 ml/min.

5.2.1.5 Injection solvent

A lot of attention has been paid to injection solvent in SFC since it can cause a significant peak distortion and thus, influence the overall performance of the chosen method [113]. In theory, sample diluent interacts not only with an analyte but with a stationary phase as well. Generally, weak solvents, such as alkanes or at least their blends with higher polarity solvents, are recommended. Monosaccharides and uronic acids used in this work are insoluble in non-polar solvents and only sparingly soluble in alcohols. We tested water/methanol mixture (1:1, v/v), pure water and water with 0.2 M TFA with equally good results on C18SB and BEH columns. This fact might be explained by a small injection volume and decreased interaction of polar sample solvent with stationary phase when water-rich modifier is used comparing to injection into pure CO_2.

5.2.2 Analysis of plant gums

We selected three plant gums most widely used as artistic binders: gum Arabic, cherry gum and gum tragacanth. Raw plant gums were subjected to MAH and analyzed on BEH and C18SB columns. Gum Arabic contained Ara, Rha, Gal, Glc and small amount of Fru and Xyl.

Cherry gum contained Ara, Xyl, Gal and small amount of Rha. Ara, Xyl, Fuc, Glc and small amount of Gal were found in gum tragacanth. Representative chromatograms are shown in Appendix B, **Fig. B5**. To our surprise, neither Man nor uronic acids were found in plant gum hydrolysates. Verification of a standard mixture before and after MAH treatment showed no degradation or significant differences in chromatograms, only ratio of anomers slightly differed. Absence of uronic acids can be explained by several factors: weak sensitivity of detection in negative mode (two orders of magnitude lower, **Fig. 5.8**), incomplete hydrolysis (due to lower temperature comparing to the literature [74]) and possible formation of lactones immediately after hydrolytic release [78]. In general, uronic acids might be involved into very resistant glycosidic linkages and even drastic conditions do not ensure complete hydrolysis of acid-containing polysaccharides (2 M H_2SO_4, 100 ºC, several hours) [78,137]. Concerning Man, its content in cherry gum can be as low as 0.3% [55]. As it was shown in the literature, gums which contained β-linked Man required longer MAH time [138]. Nevertheless, in our case longer hydrolysis time (up to 15 min) did not show traces of Man, GlcA or GalA. Taking into account a large number of existing plant cultivars and possible differences in monosaccharide composition, more samples from different suppliers are needed to make a final conclusion.

For more precise classification quantitative data on each monosaccharide was necessary. Since separation of some monosaccharides was imperfect (Ara and Xyl, Gal and Glc on BEH, Ara and Xyl on C18SB), their individual quantitation was complicated. In this case we used their summed relative peak areas and areas of well separated anomers (**Tab. 5.2** and **5.3**). Absolute values were different for BEH and C18SB. The latter revealed 2-3 times higher content of Glc and Gal in analyzed samples. Despite the absence of uronic acids and Man, results from C18SB were consistent with numbers published in the literature within the accuracy range (**Tab. 5.4** and **5.5**) [55,83]. Direct comparison of numerical values might not be indicative since calibration dependence must be established for each monosaccharide.

5.2.3 Analysis of aquarelles and archaeological sample

To test the real-life performance of our method a commercial set of high-grade watercolors and a sample of paint from ceramic fragments dated to the 14th century were used. Ara and Glc were the main monosaccharide components found in all aquarelle hydrolysates. Small amount of Rha and Gal was also present. Analysis on the BEH column was generally less sensitive and no traces of Rha were detected in contrast to C18SB which clearly showed

presence of Rha (**Tab. 5.2** and **5.3**). Absence of Rha on chromatograms from the BEH column might be explained by the lower peak height due to several times slower gradient, comparing to C18SB, and separation of anomers.

Table 5.2 BEH, average percentage of the total peak area.

Sample	Ara+Xyl total	Ara 2nd peak	Xyl 2nd peak	Rha total	Fuc total	Gal+Glc total	Glc 2nd peak	Gal 2nd peak	Fru
Arabic	**62.8**	**25.7**	**0.0**	**21.8**	**0.0**	**15.2**	**0.4**	**4.9**	**0.3**
SD	2.9	3.8	0.0	1.2	0.0	1.7	0.1	1.1	0.04
RSD, %	4.5	14.7	-	5.3	-	11.5	19.6	21.4	15.7
Cherry	**96.6**	**21.7**	**0.7**	**0.8**	**0.0**	**2.7**	**0.0**	**1.7**	**0.0**
SD	0.7	1.6	0.2	0.1	0.0	0.6	0.0	0.4	0.0
RSD, %	0.7	7.2	23.3	6.4	-	23.3	-	25.6	-
Tragacanth	**92.5**	**26.3**	**8.4**	**0.0**	**4.2**	**3.2**	**1.0**	**0.2**	**0.0**
SD	0.6	5.8	1.4	0.0	0.4	0.2	0.2	0.0	0.0
RSD, %	0.6	21.8	17.0	-	9.1	7.2	23.2	2.4	-
Ultramarine	**62.0**	**9.6**	**0.0**	**0.0**	**0.0**	**38.0**	**18.9**	**1.5**	**0.0**
SD	5.8	2.9	0.0	0.0	0.0	5.8	2.7	0.1	0.0
RSD, %	9.3	30.1	-	-	-	15.2	14.5	9.8	-
Sienna	**56.9**	**22.2**	**0.0**	**0.0**	**0.0**	**43.1**	**18.6**	**0.2**	**0.0**
SD	2.6	5.3	0.0	0.0	0.0	2.6	7.5	0.05	0.0
RSD, %	4.6	23.8	-	-	-	6.1	40.4	25.5	-
Sap green	**62.1**	**12.0**	**0.0**	**0.0**	**0.0**	**37.9**	**20.7**	**1.3**	**0.0**
SD	0.8	5.2	0.0	0.0	0.0	0.8	3.0	0.7	0.0
RSD, %	1.3	43.2	-	-	-	2.1	14.6	51.9	-

Table 5.3 C18SB, average percentage of the total peak area.

Sample	Ara+Xyl total	Ara 2nd peak	Xyl 1st peak	Rha total	Fuc total	Gal+Glc total	Glc	Gal	Fru
Arabic	**46.9**	**17.0**	**1.7**	**19.8**	**0.0**	**32.5**	**4.1**	**28.4**	**2.1**
SD	5.2	4.2	0.4	1.7	0.0	5.0	0.3	5.1	1.1
RSD, %	11.0	24.9	24.8	8.7	-	15.5	8.1	18.1	52.6
Cherry	**88.7**	**29.0**	**6.6**	**1.6**	**0.0**	**9.6**	**0.0**	**9.4**	**0.0**
SD	3.1	8.9	3.0	0.6	0.0	2.6	0.0	2.6	0.0
RSD, %	3.5	30.6	45.5	35.6	-	27.3	-	27.5	-
Tragacanth	**81.1**	**31.1**	**15.5**	**0.1**	**10.2**	**8.5**	**6.0**	**2.5**	**0.0**
SD	3.4	8.5	4.5	0.02	1.6	3.3	2.4	1.1	0.0
RSD, %	4.2	27.4	28.9	33.1	15.8	38.8	39.1	43.1	-
Ultramarine	**38.4**	**19.03**	**0.0**	**2.9**	**0.0**	**58.7**	**56.6**	**2.2**	**0.0**
SD	18.0	11.9	0.0	0.7	0.0	17.3	17.3	0.2	0.0
RSD, %	46.7	62.3	-	24.1	-	29.4	30.6	7.3	-
Sienna	**46.7**	**22.9**	**0.0**	**1.1**	**0.0**	**52.2**	**50.2**	**2.0**	**0.0**
SD	21.9	13.7	0.0	0.3	0.0	21.6	21.5	1.0	0.0
RSD, %	47.0	59.9	-	27.5	-	41.4	42.8	48.7	-
Sap green	**38.7**	**16.7**	**0.0**	**2.0**	**0.0**	**59.3**	**57.9**	**1.4**	**0.0**
SD	16.3	12.7	0.0	0.5	0.0	15.8	15.7	0.7	0.0
RSD, %	42.1	76.3	-	26.2	-	26.6	27.1	47.3	-

Sample of paint from ceramic fragments contained only Glc and Fru in 94.7:5.3 ratio (C18SB, RSD<1%). Taking into account 5 times higher peak area response factor of Glc (for the same concentration of both sugars), a real proportion of Glc to Fru should be around 80:20. Combination of these two monosaccharides may be indicative for flour, honey or fruit juice. Monosaccharides common to gum Arabic and fruit tree gums were present in aquarelles. It was impossible to attribute clearly these data to a specific plant gum.

Table 5.4 Monosaccharide composition (percentage of the total peak area) for plant gums analyzed by GC/MS, adopted from [55].

Gum	No.*	Average values – percentage of total peak area									
		Arabinose	Rhamnose	Galactose	Xylose	Fucose	Mannose	Gluc. acid	Galact. acid	Glucose	Unknown
Arabic	17	47.0	8.0	44.0	–	–	–	1.0	?**	–	–
St. Dev		8.9	4.4	4.9	–	–	–	1.0	–	–	–
%RSD		19.0	54.7	11.2	–	–	–	97.0	–	–	–
Range		**38.8–66.6**	**1.4–15.6**	**31.9–49.8**	–	–	–	**0.00–4.4**			
Tragacanth	13	50.1	–	12.6	27.4	4.5	–	–	0.3	3.2	1.9
St. Dev		8.8	–	2.4	10.8	2.0	–	–	0.3	3.1	1.5
% RSD		17.6	–	19.2	39.3	44.7	–	–	104.0	96.5	75.6
Range		**34.4–72.2**	–	**7.1–20.1**	**5.0–45.6**	**0.0–8.8**	–	–	**0.0–1.3**	**0.0–23.7**	**1.0–4.4**
Prunus	6	61.2	–	31.0	7.2	–	0.3	–	–	0.3	–
St. Dev		7.9	–	6.7	4.1	–	0.2	–	–	0.4	–
%RSD		13.0	–	21.7	57.4	–	73.1	–	–	126.7	–
Range		**51.0–68.4**	–	**18.5–40.2**	**0.0–13.6**	–	**0.0–0.6**	–	–	**0.0–1.0**	–

Table 5.5 Average relative sugar percentage content in plant gums obtained with different GC/MS procedures and 99% confidence interval, adopted from [83].

Gum	Relative percentage content (%)										Analytical procedure
	xylose	arabinose	rhamnose	fucose	galacturonic acid	glucuronic acid	fructose	glucose	mannose	galactose	
arabic	0	28 ± 4	14 ± 2	0	0	12 ± 3	-	0	0	45 ± 5	DCCI
	0	37 ± 3	18 ± 3	0	-	-	0	0	0	44 ± 5	GCI
cherry	6 ± 3	46 ± 10	1 ± 1	0	0	7 ± 7	-	0	3 ± 2	34 ± 14	DCCI
	11 ± 1	50 ± 6	2 ± 1	0	-	-	0	1 ± 1	2 ± 2	34 ± 6	GCI
tragacanth	22 ± 5	34 ± 6	2 ± 1	8 ± 2	8 ± 9	1 ± 1	-	12 ± 6	0	12 ± 2	DCCI
	24 ± 3	45 ± 5	1 ± 0	11 ± 3	-	-	1 ± 0	8 ± 3	0	9 ± 2	GCI

5.2.4 Classification of plant gums and aquarelles

5.2.4.1 Classification on the basis of monosaccharide peak area ratios

Since raw numbers were not much indicative, we tried to classify plant gums and watercolor samples on the basis of their monosaccharide ratios: Rha to (Ara+Xyl), Fuc to (Ara+Xyl) and Gal to (Ara+Xyl) [73]. Unusually high values for Glc found in samples may indicate presence of other saccharide materials. Therefore, Glc was omitted from comparison.

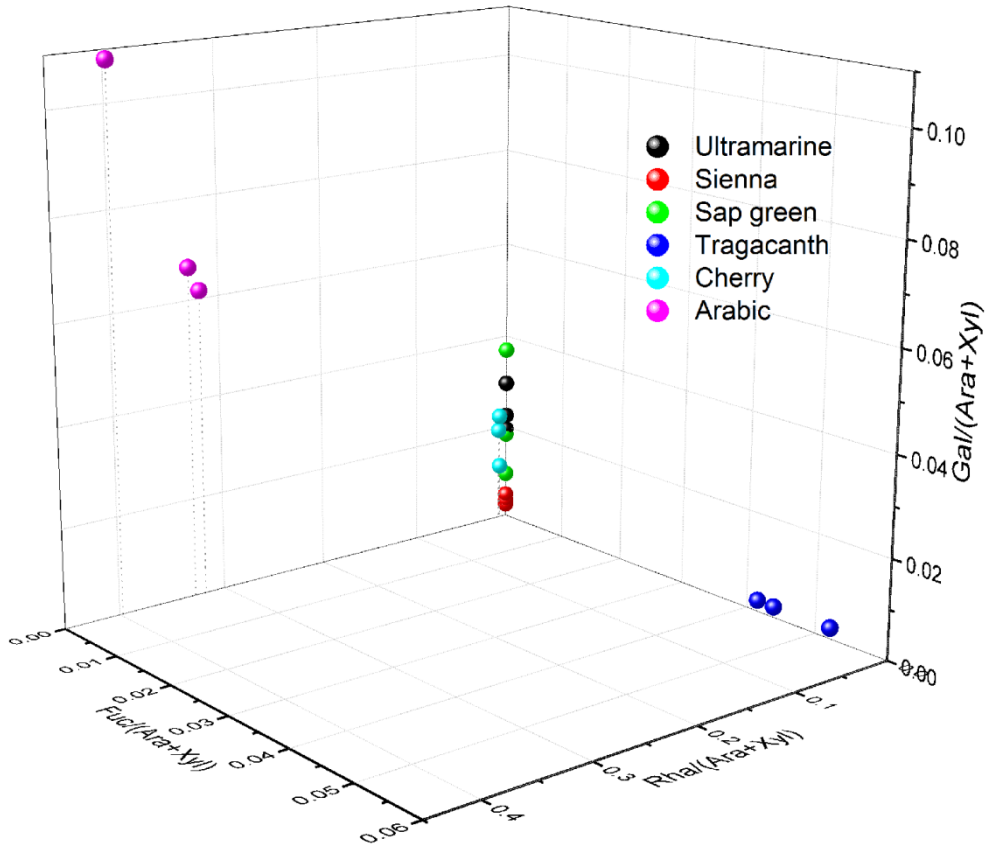

Figure 5.9 Classification of plant gums and aquarelle samples based on SFC/MS peak area ratios on the BEH column.

Table 5.6 Averaged monosaccharide peak area ratios, BEH.

Sample	Rha/ (Ara+Xyl)	RSD, %	Fuc/ (Ara+Xyl)	RSD, %	Gal/ (Ara+Xyl)	RSD, %
Arabic	0.35	10.1	0.0	-	0.08	26.5
Cherry	0.01	6.9	0.0	-	0.02	26.1
Tragacanth	0.0	-	0.05	9.8	0.002	2.2
Ultramarine	0.0	-	0.0	-	0.02	17.8
Sienna	0.0	-	0.0	-	0.004	25.8
Sap green	0.0	-	0.0	-	0.02	53.1

Results from the BEH column were lacking Rha in watercolors. Nevertheless, classification was still possible (**Fig. 5.9, Tab. 5.6**). Samples fell within the group of cherry gum. Results from the C18SB column showed distribution of plant gums similar to BEH (**Fig. 5.10**) and literature data (**Fig. 5.11**) [73]. Moreover, numerical values were in agreement with

statistics from the same literature source (compare **Tab. 5.7** and **5.8**). It is worth noting, that our gum Arabic sample occupied position similar to commercial gum Arabic from the cited source (**Fig. 5.11**). As authors suggest, differences between raw and commercial gums may be caused by different provenience or botanical species.

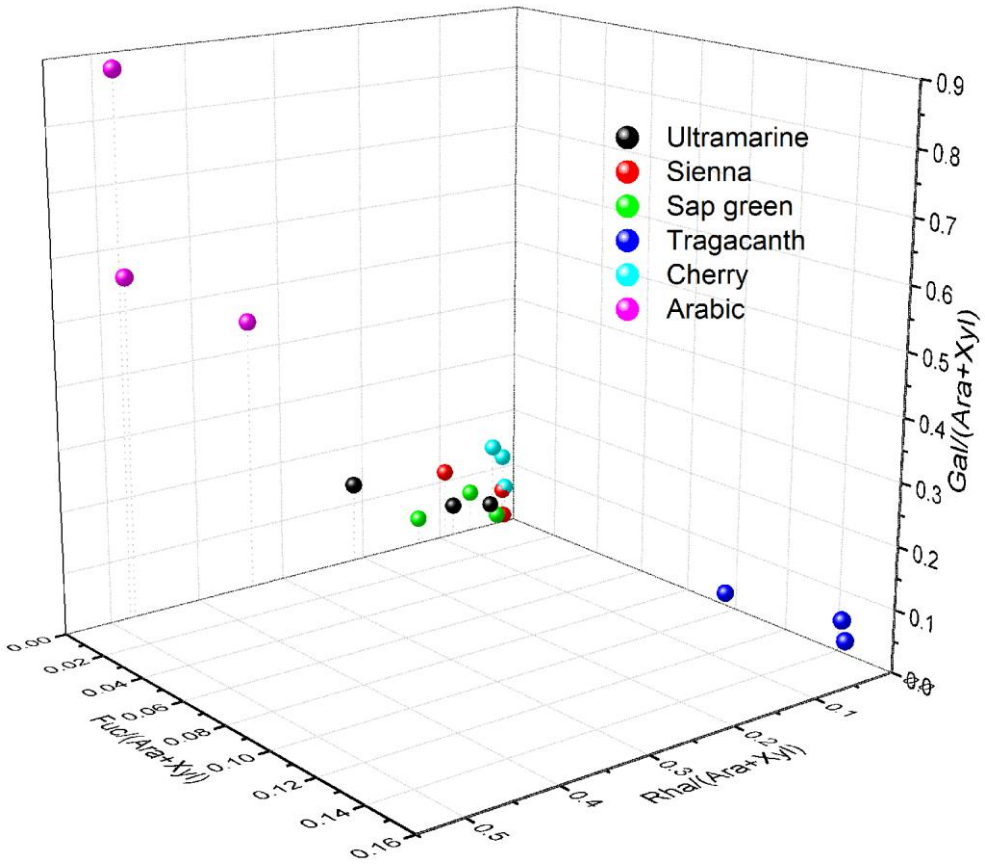

Figure 5.10 Classification of plant gums and aquarelle samples based on SFC/MS peak area ratios on the C18SB column

Table 5.7 Averaged monosaccharide peak area ratios, C18SB.

Sample	Rha/ (Ara+Xyl)	RSD, %	Fuc/ (Ara+Xyl)	RSD, %	Gal/ (Ara+Xyl)	RSD, %
Arabic	0.43	15.3	0.0	-	0.63	29.2
Cherry	0.02	38.7	0.0	-	0.11	30.3
Tragacanth	0.001	37.9	0.13	17.3	0.03	47.9
Ultramarine	0.11	69.8	0.0	-	0.07	56.2
Sienna	0.04	90.8	0.0	-	0.06	68.1
Sap green	0.07	62.2	0.0	-	0.04	50.3

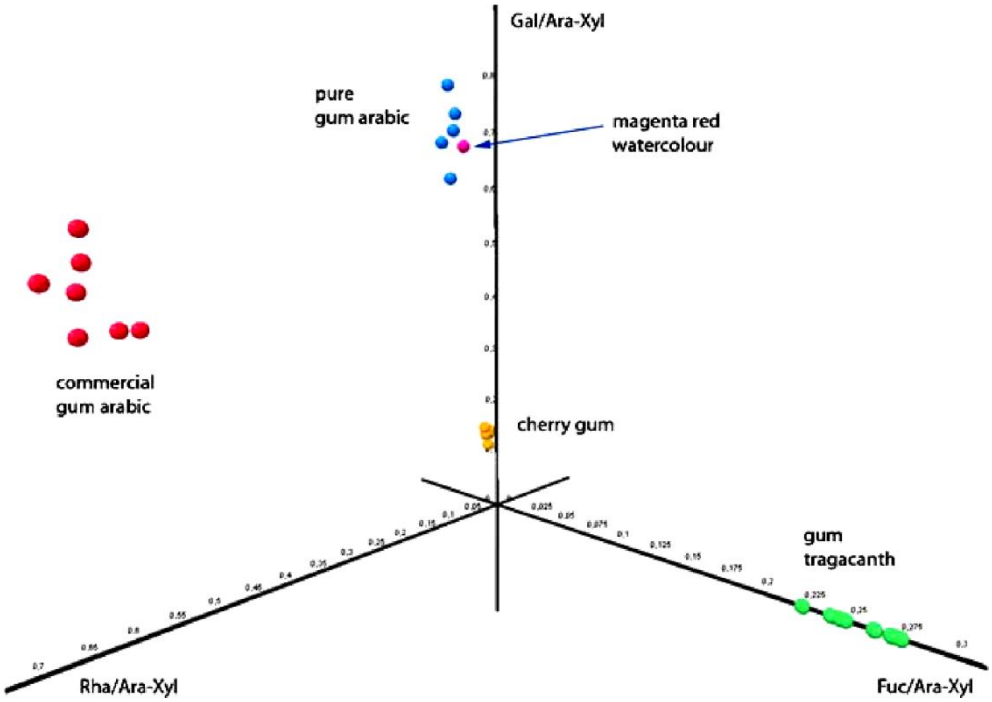

Figure 5.11 Classification of plant gums based on average GC/MS peak area ratios, adopted from [73]

Table 5.8 Averaged monosaccharide peak area ratios, GC/MS, adopted from [73].

Sample	Rha/ara–xyl	RSD%	Gal/ara–xyl	RSD%	Fuc/ara–xyl	RSD%
Gum arabic	0.10	10.73	0.70	8.21		
Commercial gum arabic	0.62	4.99	0.46	10.96		
Gum tragacanth					0.25	6.99
Cherry gum	0.02	21.02	0.15	8.93		

5.2.4.2 Classification on the basis of principal component analysis

PCA analysis was based on the relative peak areas of monosaccharides as variables. For the sake of comparison, PCA including all variables and PCA without Glc and Fru data were performed. In both cases data from BEH and C18SB columns enabled clear grouping of plant gum samples. Aquarelles did not overlap with any of the plant gums and formed a separate group. Their position was correlated with Glc on the loading plot (**Fig. 5.12, 5.14)**. After exclusion of Glc which may be present due to contamination or another saccharide material, group of aquarelles moved towards the cherry gum (**Fig. 5.13, 5.15)**. A binder from another source could be used for manufacturing of aquarelles, most likely another fruit tree belonging to *Prunus* sp. More gum samples from different sources are needed for objective comparison.

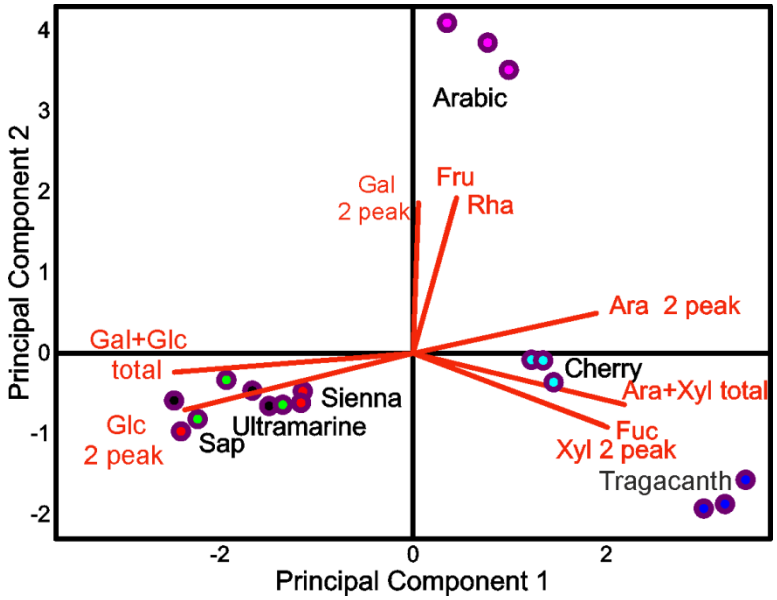

Figure 5.12 PCA biplot of plant gums and aquarelles based on all variables, BEH column. PC1 and PC2 account for 44.8% and 44.5% of the total variance, respectively.

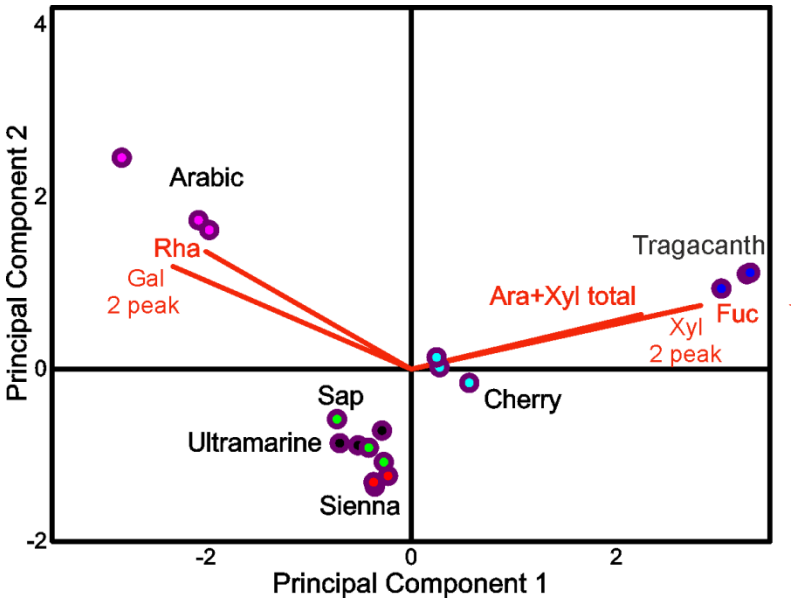

Figure 5.13 PCA biplot of plant gums and aquarelles based on selected variables, BEH column. PC1 and PC2 account for 58.0% and 28.8% of the total variance, respectively.

PCA provided more precise results comparing to the classification on the basis of monosaccharide peak area ratios. In the latter case binder in the aquarelle samples might be mistakenly attributed to cherry gum. Concerning plant gum classification, the reported results are comparable with PCA based on GC/MS data (**Fig. 5.16**) [83]. This is a fact of a very high

importance since these results were obtained under different conditions using absolutely different procedures, chromatographic and detection techniques.

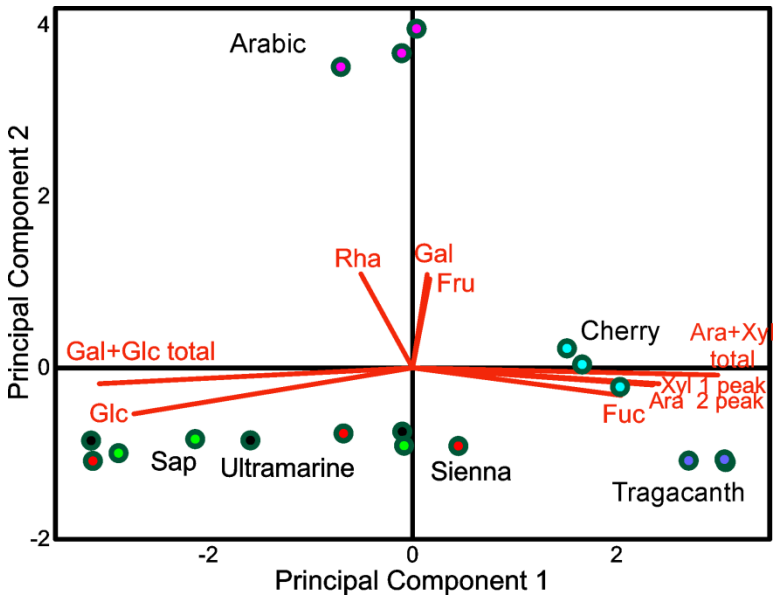

Figure 5.14 PCA biplot of plant gums and aquarelles based on all variables, C18SB column. PC1 and PC2 account for 46.8% and 34.1% of the total variance, respectively.

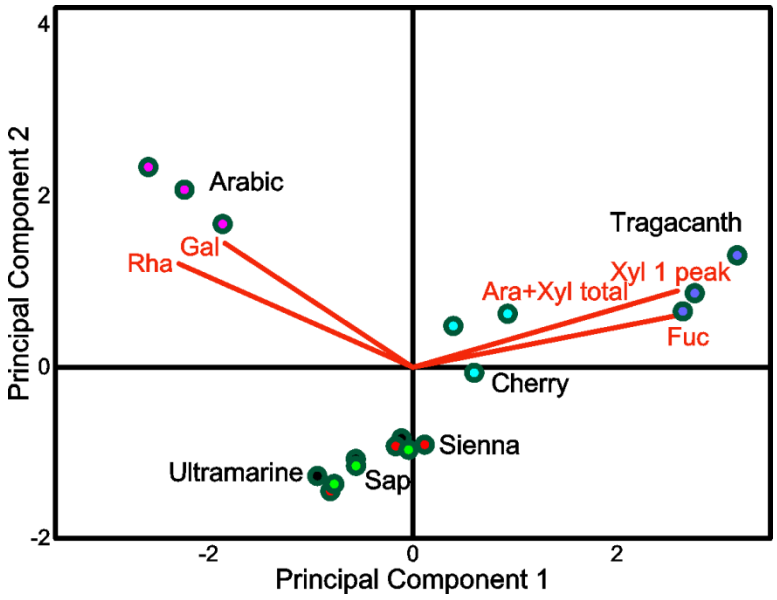

Figure 5.15 PCA biplot of plant gums and aquarelles based on selected variables, C18SB column. PC1 and PC2 account for 51.9% and 32.3% of the total variance, respectively.

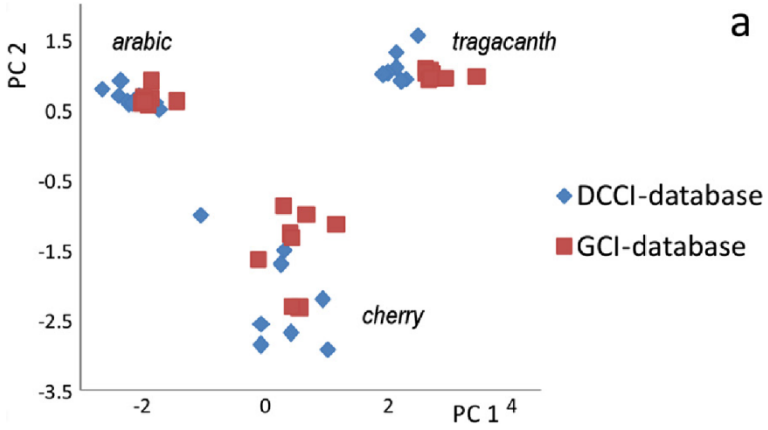

Figure 5.16 PCA score plot of the three plant gums obtained using profiles from Department of Chemistry and Industrial Chemistry of the University of Pisa, Italy (DCCI) and Getty Conservation Institute in Los Angeles, USA (GCI) databases, adopted from [83].

5.2.5 Conclusion

SFC was primarily recognized as a technique for non-polar analytes. Nevertheless, since its discovery the range of application was significantly expanded. Modifiers and additives play crucial role in the positive outcome of analysis. In this work, water with formic acid as components of modifier provided increase in the elution strength of SFC mobile phase and allowed separation of nine monosaccharides and elution of very polar sugar acids. Among the tested columns C18SB showed the best results in terms of monosaccharide separation, analysis time and sensitivity. BEH was suitable for separation of the standard mixture but demonstrated lower sensitivity, and cannot be recommended for analysis of real samples. The developed SFC/MS method was applied for examination of plant gums used as binders in painting media.

Although developed method did not indicate presence of Man and uronic acids in plant gums, analyzed samples were successfully classified by PCA on the basis of selected monosaccharide relative peak areas. Gum Arabic, cherry gum and tragacanth were clearly distinguished from each other. Aquarelles, however, did not fell in any gum group and occupied a separate zone. A binder from another source could be used for manufacturing of aquarelles, most likely a fruit tree belonging to *Prunus* sp. Sample of paint from historical ceramic fragments had different monosaccharide profile and contained only Glc and Fru. This combination may indicate use of flour, honey or fruit juice.

Identification of plant gum binders in historical artifacts and artworks is a challenging task. There is no perfect method for analysis of plant gums since each has some limitations.

Classification on the basis of percentage of sugars is not always possible. Classification on the basis of the main peak ratios can be used only with data obtained under identical experimental conditions. As already reported in the literature [83] and once again confirmed by this work, used technique and analytical parameters significantly affect peak area ratios. However, use of multivariate data analysis, such as PCA, reveals differences and similarities between the samples and allows comparison of chromatographic profiles obtained under different analytical conditions.

5.3 Development of SFC/MS method for analysis of polar designer drugs

Part of materials shown in this section has been accepted for publication:

V. Pauk, V. Žihlová, L. Borovcová, V. Havlíček, K. Schug, K. Lemr, Fast Separation of Selected Cathinones and Phenylethylamines by Supercritical Fluid Chromatography, *J. Chromatogr. A* (2015).

5.3.1 Mass spectrometric detection

To achieve the highest ionization efficiency of NDDs influence of different make-up liquids was tested: methanol; methanol : water 50:50 (v/v); water; all with and without 1% formic acid or 20 mM ammonium hydroxide. Differences were not significant, though pure methanol provided slightly better results in terms of absolute intensity and S/N values for each precursor ion (data not shown).

For each compound four SRM transitions were found (see **Tab. 4.2**). In the experiments, a single most intensive specific transition was monitored, where possible. For chromatographic experiments utilizing pure standards, it is more logical to use one SRM transition per compound and maintain the highest points-per-peak value. In most cases $W_{1/2}$ was less than 3 s. Under these circumstances longer scanning times caused by monitoring of multiple transitions for each of 15 investigated compounds can result in inaccurate chromatographic peak shape (see **Fig. 5.17**).

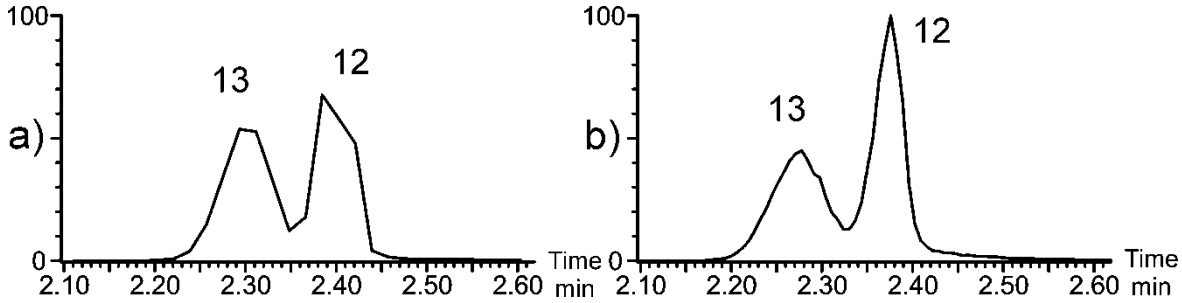

Figure 5.17 Impact of MS scan rate on chromatographic peak shape of ethylone (**12**) butylone (**13**), **a)** 39 SRM transitions, 3.7 points per peak on average; **b)** 14 SRM transitions, 10.2 points per peak on average.

We encountered four pairs of isomeric compounds that cannot be unambiguously distinguished by MS: buphedrone (**2**) and 3-MMC (**3**); 3-FMC (**5**) and flephedrone (**6**); BDB (**9**) and methedrone (**10**); ethylone (**12**) and butylone (**13**) due to the interfering SRM transitions. Unexpectedly, 2-EP stationary phase demonstrated very high background noise

for 2C-H transition present in blank and sample injections. S/N ratios for 2C-H were roughly 30-60 times worse than for other compounds.

5.3.2 Selection of additives

Methanol without additives delivered distorted wide peaks ($W_{1/2}$ around 10 s) for all compounds on all columns (Appendix C, **Fig. C1**). Moreover, 2C-B was not eluted from C18SB, 2C-H was not eluted from 2-EP and from 3 to 9 compounds were strongly retained on the Fluoro-Phenyl column, depending on the temperature. With 2% of water in methanol all compounds were eluted, but peak shapes remained poor (**Fig. C2**). Peak broadening can be explained by dynamic equilibrium between neutral and protonated form of basic drugs present at these conditions and interaction with stationary phase support.

Formic acid, as expected, resulted in unacceptable chromatograms (**Fig. C3**) due to apparent protonation of aminogroup and thus, supporting secondary electrostatic interactions of analytes with stationary phases but one exception – Fluoro-Phenyl. In the latter case we observed 11 enough distinguished peaks at 60 °C. It is known that another type of retention mechanism takes place for this stationary phase, namely π-π interactions between aromatic rings [139]. However, it does not explain the unusual behavior in the presence of formic acid. Positively charged hybrid surface might have greater impact on improved peak shapes for protonated analytes in this case.

Ammonium hydroxide and its salts, formate and acetate, delivered significantly better results for all stationary phases (Appendix C, **Fig. C4-C6**, **Tab. C2-C5**). Using these additives all investigated compounds were eluted in 3.5 minutes or less and most of the peaks had Gaussian shape (see **Fig. 5.18** as an example). Chromatogram improvement was evident already at 5 mM concentration of the additives and best results were reached using 20 mM concentrations (**Tab. C2, C3, C6, C7**). Addition of 2% water to ammonium formate modifier allowed to resolve BDB (**9**) and methedrone (**10**) on the Fluoro-Phenyl column and improved separation of ethylone (**12**) and butylone (**13**) on C18SB. This positive influence can be explained by partial neutralization of methylcarbonic acid, which is known to be formed under supercritical conditions [140], by hydroxide and possible blockage of active sites on stationary phases by ammonium ions. As it was shown by NMR studies, after exposure to ammonium additives protons of active silanol groups on bare silica phase are replaced by ammonium cations to a significant extent [141]. Thus, an ion-exchange mechanism takes place when ammonium additives are used to elute positively charged analytes.

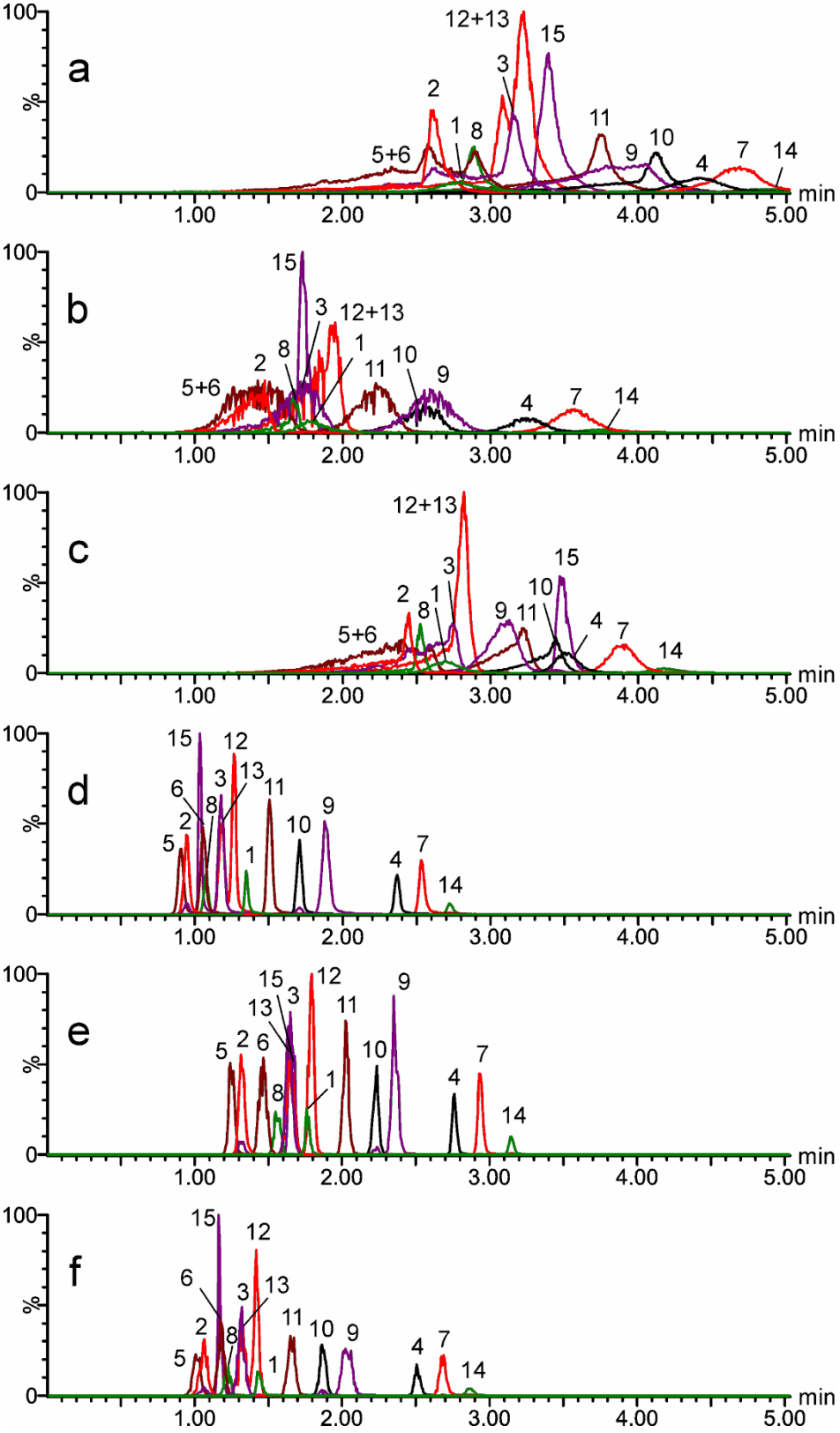

Figure 5.18 The influence of additives on separation of cathinones and phenylethylamines, BEH, 40 ºC: **a)** MeOH without additives, **b)** MeOH with 2% H_2O, **c)** 20 mM HCOOH, **d)** 20 mM NH_4OH, **e)** 20 mM $HCOONH_4$, **f)** 20 mM CH_3COONH_4.

Ammonium hydroxide decreased retention in comparison to ammonium formate, but retention factors were highly correlated on all used columns (**Fig. 5.19**). These additives were compatible with mass spectrometric detection, improved peak shape, provided faster elution of compounds but did not change significantly the selectivity of separation.

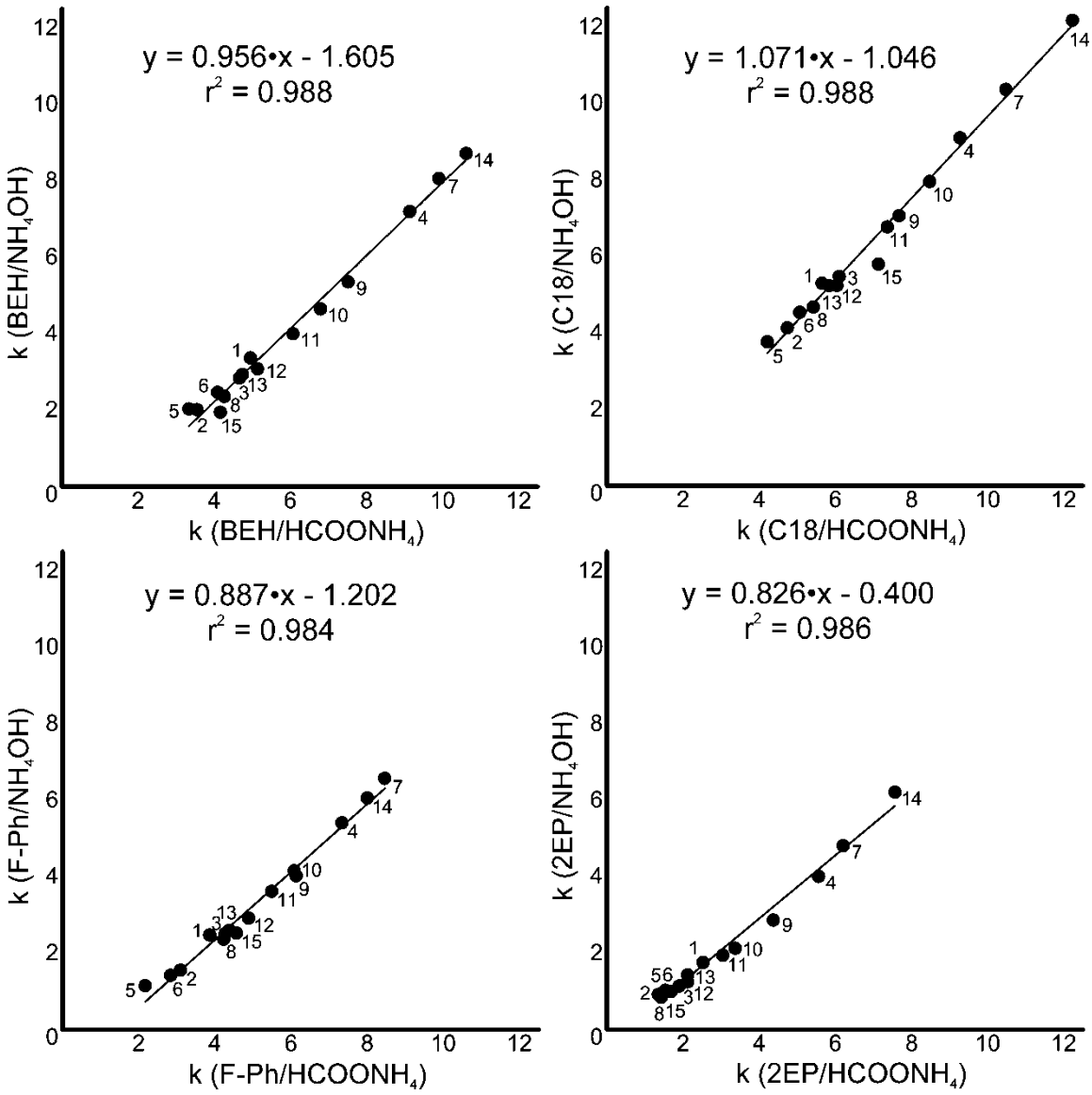

Figure 5.19 The correlation of retention factors of cathinones and phenylethylamines on investigated stationary phases at 50 ºC for 20 mM NH_4OH and $HCOONH_4$ additives.

Nevertheless, some changes of elution order, peak symmetry, and separation of isomers were noticed. For instance, naphyrone (**15**) eluted the first using ammonium hydroxide but as the fourth component in ammonium formate on BEH. The elution order of BDB (**9**) and methedrone (**10**) was reversed on the Fluoro-Phenyl column when ammonium hydroxide was

substituted by ammonium formate. Using the BEH column, ammonium hydroxide provided symmetrical peaks for all 15 compounds, while ammonium formate provided symmetrical peaks only for 10. A similar trend was observed for the Fluoro-Phenyl column. Using C18SB and 2-EP columns, the number of symmetrical peaks was comparable for both additives. Concerning the separation of isomeric pairs, ammonium salts ensured slightly better resolution than ammonium hydroxide, with the most evident difference for ethylone (**12**) and butylone (**13**) on all columns except for 2-EP (see **Tab. C6-C9**).

Ammonium additives showed positive impact on detection sensitivity. For the most of substances peak heights and S/N ratio were best at 20 mM concentration. All three additives demonstrated comparable results. Typically, peak heights were higher than 10^6 and S/N ratios were in the range 1000-10000 for 1 ng injection on BEH and Fluoro-Phenyl columns, with the lowest values for 2C-B.

5.3.3 Column temperature

Generally, setting the column temperature in the 40-60 °C range influenced resolution and peak shape less than the additives, but proper selection of temperature was still important (see **Tab. C2-C9**). The separation of ethylone (**12**) and butylone (**13**) deteriorated with increasing temperature and was completely lost on the BEH column at 60 °C for ammonium hydroxide and ammonium acetate. The number of symmetrical peaks was lower at 40 °C on the C18SB column for ammonium hydroxide and its salts. Increase in temperature from 40 to 60 °C resulted in 6-7% or 8-9% longer analysis times on 2-EP and Fluoro-Phenyl columns, respectively. The number of symmetrical peaks decreased with increasing temperature on the same columns for ammonium hydroxide and ammonium acetate. Despite these minor drawbacks 60 °C was the only acceptable temperature for separation of BDB (**9**) and methedrone (**10**) on the Flouro-phenyl column with ammonium hydroxide. Lower temperature (35 °C) resulted in increased retention and spreading of early eluting peaks ((**2**), (**3**), (**5**), (**6**), (**8**), (**12**), (**13**), data not shown) on BEH with ammonium hydroxide.

5.3.4 Evaluation of stationary phases

Primary criterion for choosing the best stationary phase and the most suitable conditions was resolution of isomeric compounds, since these cannot be unambiguously distinguished by MS despite selective SRM mode. Number of chromatographically resolved peaks, resolution between critical pairs (including MS-distinguishable), peak asymmetry and analysis time were

the next evaluation parameters. The most noteworthy results were summarized (Appendix C, **Tab. C2-C5**) and best results for each stationary phase were chosen (**Tab. 5.9**). Resolution between isomers for different columns and conditions is listed in **Tab. C6-C9**, Appendix C.

The first criterion was fulfilled only by 2 phases: BEH and Fluoro-Phenyl. Three pairs showed resolution greater than or equal to 2.0 on BEH at 40 ºC. Ethylone (**12**) and butylone (**13**) were more resolved on Fluoro-Phenyl. However, the highest resolution for this pair, as well as for BDB (**9**) and methedrone (**10**), on the Fluoro-Phenyl phase was achieved by addition of 2% water to methanolic ammonium formate. Among these the largest number of resolved peaks, 11, was observed on BEH using ammonium hydroxide and ammonium formate. C18SB failed to resolve ethylone (**12**) and butylone (**13**) regardless all investigated experimental conditions, though 2% of water with ammonium formate provided slightly better results. 2-EP did not separate 3-FMC (**5**) and flephedrone (**6**) and showed insufficient resolution for ethylone (**12**) and butylone (**13**). Best chromatograms for each stationary phase are shown in **Fig. 5.20**.

Table 5.9 Evaluation of stationary phases.

St. phase, additive	Temp., °C	Isomers			All compounds					
		$R>1$	$1>R>0.3$	$R<0.3$	$R>1$	$1>R>0.3$	$R<0.3$	Gauss.	Time, min	$W_{1/2}$, s
BEH, NH_4OH	40	4	0	0	11	2	2	15	2.87	2.51
BEH, $HCOONH_4$,	40	4	0	0	11	2	2	10	3.25	2.61
Fl-Ph, NH_4OH	60	4	0	0	10	4	1	11	2.36	1.99
Fl-Ph, $HCOONH_4$, 2% H_2O	60	4	0	0	10	4	1	10	2.62	2.34
C18, $HCOONH_4$, 2% H_2O	60	3	1	0	12	2	1	13	3.67	2.30
2-EP, NH_4OH	40	2	1	1	6	5	4	10	2.08	2.29

R – chromatographic resolution between critical pairs
Gauss. – number of Gaussian-shaped peaks, asymmetry factor in the range 0.8 – 1.4
Time – complete elution of the last peak

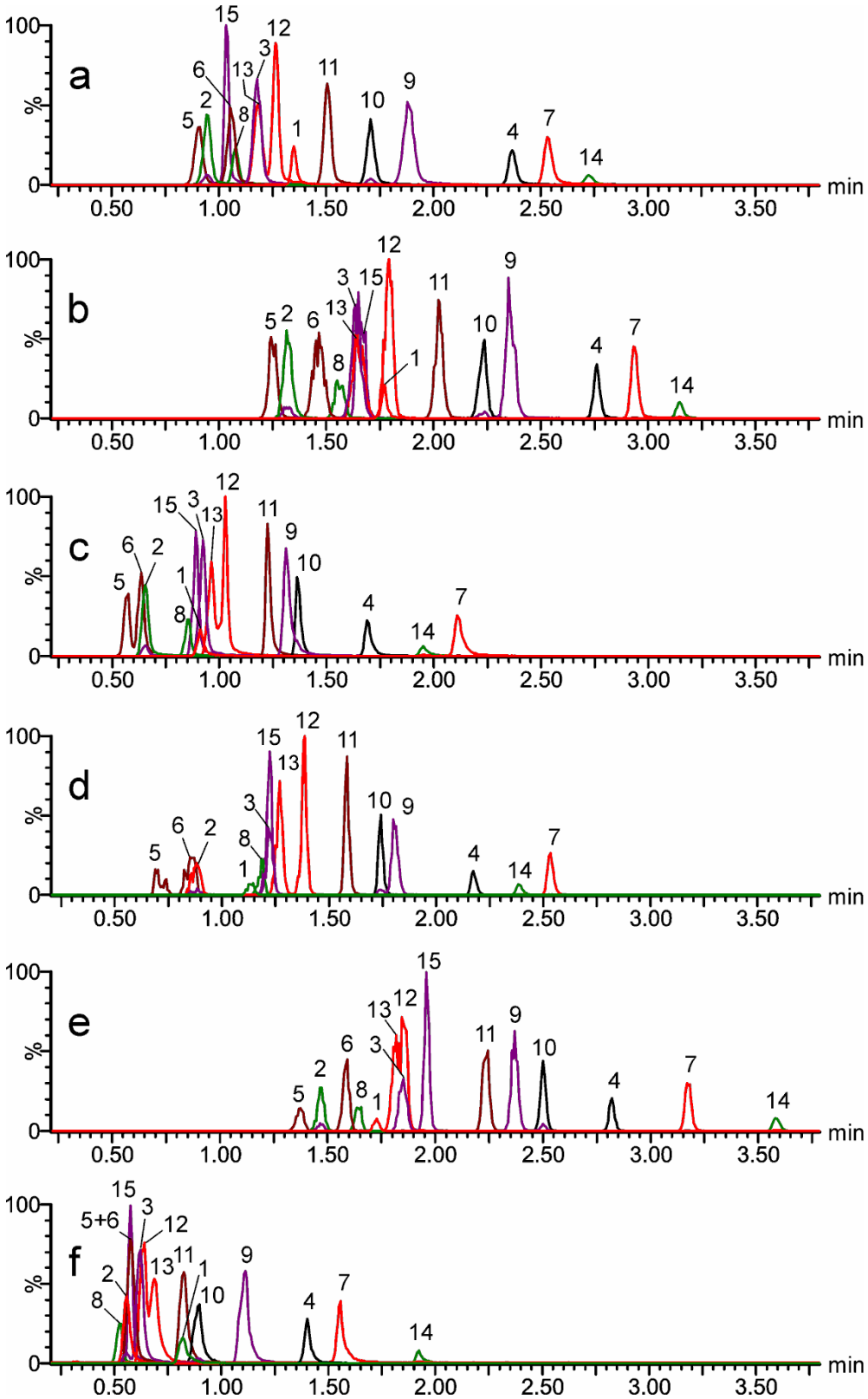

Figure 5.20 The most suitable separation conditions for an each stationary phase: **a)** BEH 40 °C, 20 mM NH_4OH; **b)** BEH 40 °C, 20 mM $HCOONH_4$; **c)** Flouro-Phenyl 60 °C, 20 mM NH_4OH; **d)** Flouro-Phenyl 60 °C, 20 mM $HCOONH_4$, 2% H_2O; **e)** C18SB 60 °C, 20 mM $HCOONH_4$, 2% H_2O; **f)** 2-EP 40 °C, 20 mM NH_4OH.

5.3.5 Speed of analysis

Under the most suitable conditions 15 drugs were separated on BEH in less than 3 min. Further increase of analysis speed was attempted by tuning initial content of the modifier, gradient slope, flow rate and ABPR. Increase of the flow rate and gradient slope reduced analysis time to 1.6 min and maintained acceptable separation of isomers (**Fig. 5.21**).

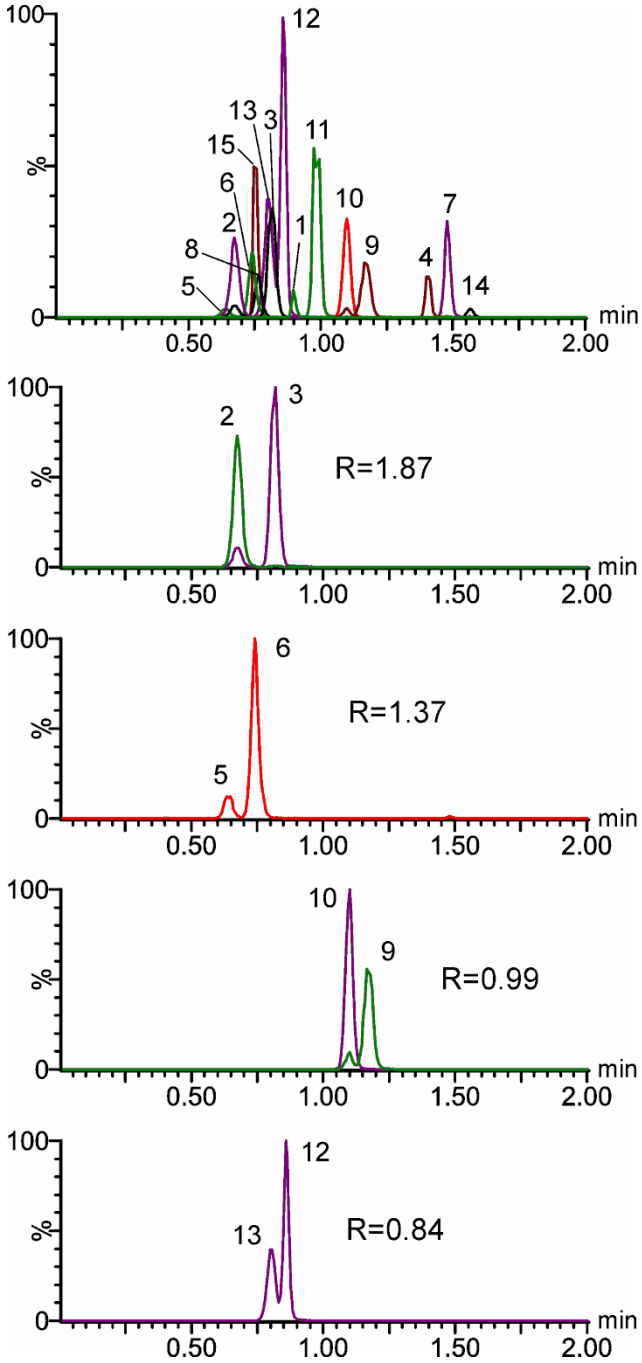

Figure 5.21 Ultra-fast analysis on the BEH column, 40 °C, 20 mM NH_4OH, 6-24% in 1.5 min, 2.9 ml/min, max. pressure 400 bar.

5.3.6 Retention correlation

All investigated stationary phases showed very similar selectivity. The retention factors (**Tab. C10**) for analytes separated on 2-EP and Fluoro-Phenyl phases correlated less (r^2=0.845, **Fig. 5.22**, **a**) than those on the other stationary phase combinations ($r^2 \geq 0.899$). The same pair rendered the most significant differences for cathinones belonging to secondary amines (r^2=0.741, **Fig. 5.22**, **b**) in comparison to other stationary phases ($r^2 \geq 0.869$). Retention factors of primary amines exhibited the lowest correlation for these phases (r^2=0.872, **Fig. 5.22**, **c**). Comparing Fluoro-Phenyl with other stationary phases, an interesting difference was observed for analytes with a halogen atom at position 4 of the aromatic ring. For instance, flephedrone and 2C-B possessing fluorine or bromine atoms, respectively, were less retained on Fluoro-Phenyl. The electronegative atoms attract electrons from aromatic rings which might decrease π-π interaction between the stationary phase and analytes. Thus, different selectivity for halogen-substituted drugs could be expected on the Fluoro-Phenyl phase. It was the only phase that retained 2C-H to a greater degree than 2C-B. Overall, a decrease in the polarity of stationary phases (2-EP>BEH>Fluoro-Phenyl>C18SB) resulted in lower selectivity for BDB (**9**) and methedrone (**10**), and a reversed elution order was observed on the C18SB phase.

We observed several structure-related correlations in chromatographic behaviour of investigated compounds common to all stationary phases (**Fig. 5.20**). Longer alkyl chain at or in close proximity to amino group caused shorter retention of cathinones ((**12**) < (**11**), (**13**) < (**11**), (**8**) < (**3**), (**2**) < (**1**)), as well as of phenylethylamines ((**9**) < (**4**) < (**7**)). Phenylethylamines without an alkyl group in the α-position (2C-H (**7**) and 2C-B (**14**)) were the most retained substances regardless of the stationary phase. Therefore, accessibility of the nitrogen atom in amino group is directly related to retention of a particular substance. The presence of methylenedioxy group increased polarity and retention of cathinones ((**2**) < (**13**)). A similar effect was observed with the substitution of 4-methyl by 3,4-methylenedioxy group ((**8**) < (**12**)) in contrast to substitution of 4-methoxy by 3,4-methylenedioxy group, which decreased the actual retention ((**11**) < (**10**)). Fluorine-substituted cathinones eluted faster than methyl ((**5**) < (**3**)), methoxy ((**6**) < (**10**)) or methylenedioxy substituted ones ((**6**) < (**11**)). In general, for very similar structures, a substance with higher log *P* and lower pK_a (less polar) should elute earlier. However, greater retention was observed for a 4-methoxy (**10**) contrary to a 3,4-methylenedioxy derivative (**11**) despite decrease of log *P* and increase of pK_a values. The

elution order of (**7**) and bromine-substituted (**14**) also differed from the predicted behaviour, except in the case of the Fluoro-Phenyl phase. Isomeric pairs with different structures ((**2**) and (**3**), (**9**) and (**10**)) can be resolved on all stationary phases while separation of positional isomers possessing very similar characteristics ((**5**) and (**6**), (**12**) and (**13**)) was difficult, especially on C18SB and 2-EP phases.

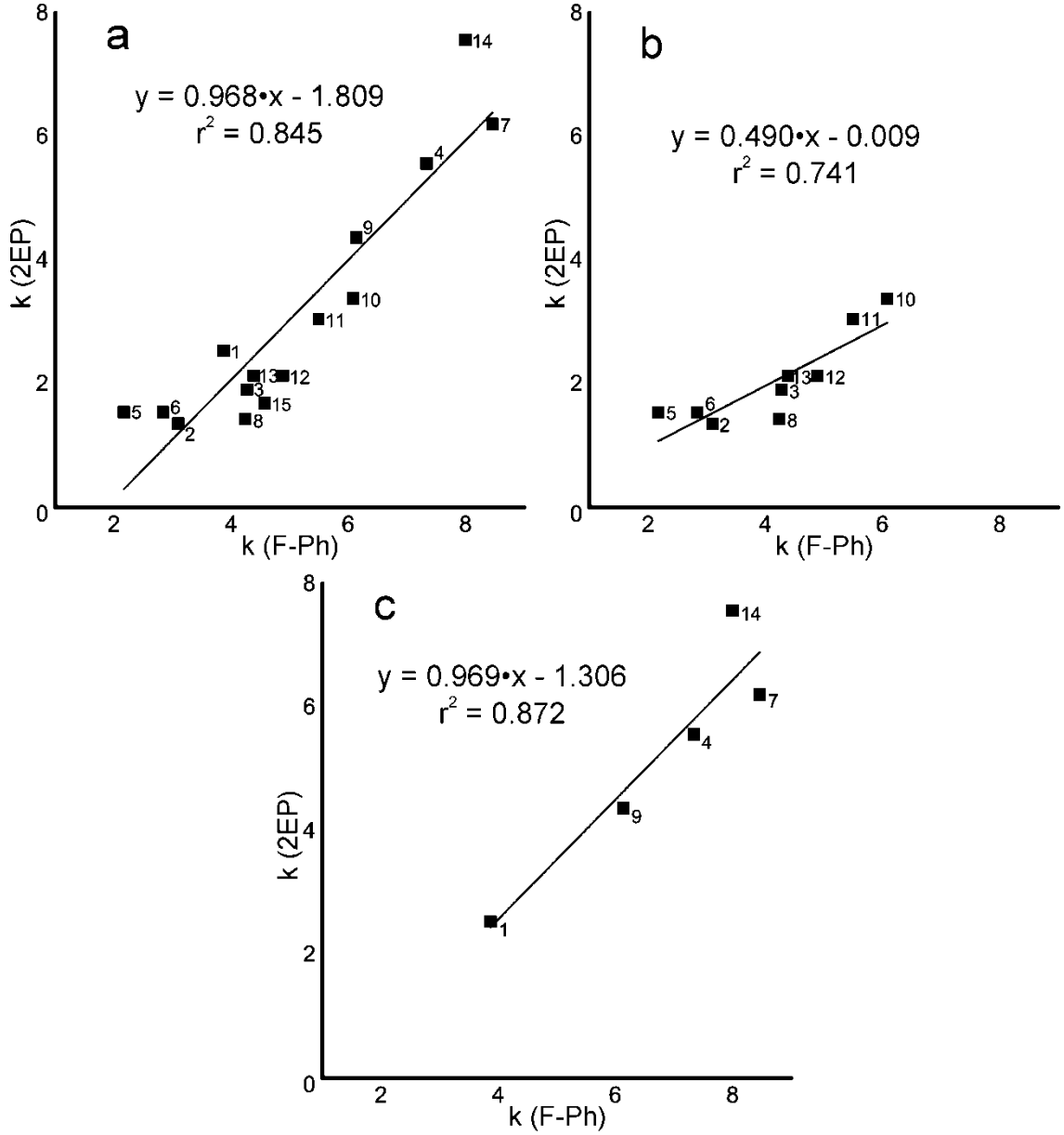

Figure 5.22 The correlation of retention factors calculated for 2-EP and Fluoro-Phenyl columns, at 50 ºC, 20 mM $HCOONH_4$: **a)** all substances, **b)** secondary amines, **c)** primary amines.

5.3.7 Conclusion

This is the first report on the application of UHPSFC for separation of polar synthetic cathinones and strongly basic phenylethylamines. The four stationary phases, BEH silica, BEH 2-ethylpyridine, CSH Fluoro-Phenyl and HSS C18SB were tested under various conditions with different mobile phases. Highly correlated retention factors were obtained using different additives (ammonium formate and ammonium hydroxide) as well as stationary phases. However, some differences in elution order, peak symmetry and separation of isomers were noticed. Roughly, substances with higher log *P* and lower pK_a eluted faster. More detailed evaluation revealed some structure features influencing retention. Especially, the accessibility of a nitrogen atom in an amino group had the greatest impact on retention time of a particular substance.

The best separation results were achieved using BEH phase at 40 °C and 20 mM ammonium hydroxide or ammonium formate as modifier. Alternatively, CSH Fluoro-Phenyl can be used at 60 °C with 20 mM ammonium hydroxide or ammonium formate and 2% water in methanol as modifier. Under these conditions, the four isomeric pairs were sufficiently separated and occasional co-eluting species were easily distinguished by MS. The analysis took less than 3.3 minutes and could even be reduced to 1.6 minutes on the BEH column for ultra-fast screening of NDDs.

6 CONCLUSION

Three mass spectrometric methods were developed for forensic applications. The first one, FIA/ESI-MS protocol, proved to be useful in detection of important historical pigments – Prussian blue (PB) and indigo. To our best knowledge, this is the only ESI-MS method for simultaneous identification of PB and indigo by the moment of publication. This method is rapid, simple, sensitive and does not require chromatographic separation. It offers indigotin LOD comparable to HPLC/MS (absolute LOD of PB was not reported in the literature). It represents a useful alternative to other methods applied in simultaneous analysis of both pigments, such as LDI-MS, infrared or Raman spectroscopy and SERS. The developed method showed presence of PB in the microsample of 'Crucifixion' which could exclude Middle Ages origin of the painting. Since the painting is a palimpsest with cracks, analysis of other samples from different locations is suggested to confirm PB in both layers.

The second, SFC/MS method, was applied for analysis of saccharide binders in painting medium. Water and formic acid as components of modifier increased the elution strength of SFC mobile phase and allowed separation of nine monosaccharides and elution of very polar sugar acids. Among the tested columns, C18SB showed the best results in terms of monosaccharide separation, analysis time and sensitivity. Sample of paint from the 14^{th} century ceramic fragments contained Glc and Fru which may indicate usage of honey, flour or fruit juice as a binder. Profile of commercial aquarelles was compared with plant gums. Gum Arabic, cherry gum and tragacanth were clearly distinguished from each other by PCA. Aquarelles did not fell in any gum group and occupied a separate zone. An alternative plant source of the binder used in watercolors is suggested, most likely a fruit tree belonging to *Prunus* species.

The third method was focused on analysis of NDDs. This is the first report on the application of UHPSFC for separation of polar synthetic cathinones and strongly basic phenylethylamines. SFC conditions were extensively investigated and general trends in chromatographic behavior of polar drugs were established. Retention factors were highly correlated on all stationary phases using ammonium based additives. In general, substances with higher log *P* and lower pK_a eluted faster. The accessibility of a nitrogen atom in an amino group had the greatest impact on retention time of a particular substance. Efficient separation of four isomeric pairs and most of remaining analytes (fifteen compounds in total)

was achieved in less than 3 minutes on BEH phase at 40 °C using 20 mM ammonium hydroxide or ammonium formate in methanol as modifier. Alternatively, CSH Fluoro-Phenyl can be used at 60 °C with 20 mM ammonium hydroxide or ammonium formate and 2% water in methanol as modifier. ESI ionization with a triple quadrupole analyzer in SRM mode provided an additional dimension for differentiation and sensitive detection of all investigated substances. The analysis time was further reduced to 1.6 minutes on the BEH column for ultra-fast screening of NDDs.

This work shows versatility and usefulness of mass spectrometric detection for forensic applications. Its real potential can be uncovered introducing a fast and efficient separation step such as SFC. It offers higher selectivity, improvement in sensitivity and analysis time comparable with FIA.

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LIST OF SYMBOLS AND ABBREVIATIONS

ABPR	automatic backpressure regulator
APCI	atmospheric pressure chemical ionization
CE	capillary electrophoresis
CI	confidence interval
DART	direct analysis in real time
ELISA	enzyme-linked immunosorbent assay
ELSD	evaporative light scattering detector
FIA	flow injection analysis
FT-ICR	Fourier-transform ion cyclotron resonance
GC	gas chromatography
IR	infrared spectroscopy
HILIC	hydrophilic interaction liquid chromatography
HPLC	high-performance liquid chromatography
HRMS	high resolution mass spectrometry
k	retention factor (capacity factor in former nomenclature)
LDI	laser desorption ionization
LOD	limit of detection
log *P*	logarithm of water/octanol partition coefficient
MAH	microwave assisted hydrolysis
MALDI	matrix-assisted laser desorption ionization
MRM	multiple reaction monitoring (outdated nomenclature)
MS	mass spectrometry
M_w	weight-average molecular weight
NDD	new designer drug
NMR	nuclear magnetic resonance spectroscopy
PCA	principal component analysis
pK_a	dissociation constant
R	chromatographic resolution
r^2	coefficient of determination
RI	refractive index detector

SD	standard deviation
SERS	surface enhanced Raman spectroscopy
SFC	supercritical fluid chromatography
sp.	species
SRM	selected reaction monitoring
var.	variety
TOF	time of flight mass analyzer
UHPLC	ultra high-performance liquid chromatography
UHPSFC	ultra high-performance supercritical fluid chromatography
UNODC	United Nations Office on Drugs and Crime
UV/Vis	ultraviolet/visible spectroscopy
$W_{1/2}$	full width at half maximum of a chromatographic peak
XRF	X-ray fluorescence spectroscopy

LIST OF SUBSTANCES

AG	arabinogalactan
AGP	arabinogalactan-protein complex
2C-B	2,5-dimethoxy-4-bromophenylethylamine
2C-H	2,5-dimethoxyphenylethylamine
3-FMC	3-fluoromethcathinone
3-MMC	3-methylmethcathinone
4-FMC, flephedrone	4-fluoromethcathinone
4-MEC	4-methylethylcathinone
ACN	acetonitrile
Ara	arabinose
BDB	3,4-methylenedioxybutanphenamine
bk-MBDB, butylone	β-keto-N-methylbenzodioxolylbutanamine
bk-MDEA, ethylone	3,4-methylenedioxy-N-ethylcathinone
bk-MDMA, methylone	3,4-methylenedioxy-N-methylcathinone
bk-PMMA, methedrone	4-methoxymethcathinone
buphedrone	α-methylamino-butyrophenone
cathinone	α-aminopropiophenone
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
Fru	fructose
Fuc	fucose
Gal	galactose
GalA	galacturonic acid
Glc	glucose
GlcA	glucuronic acid
GP	glycoprotein
hex	hexose
Man	mannose
MDA	3,4-methylenedioxyamphetamine
MDMA	3,4-methylenedioxymethamphetamine

MDPV	3,4-methylenedioxypyrovalerone
MeOH	methanol
MSA	methanesulfonic acid
naphyrone	naphthylpyrovalerone
PB	Prussian blue
Rha	rhamnose
Rib	ribose
TFA	trifluoroacetic acid
THF	tetrahydrofuran
Xyl	xylose

CURRICULUM VITAE

Name: Volodymyr Pauk

Year and place of birth: 1988, Uzhhorod, Ukraine

E-mail: volodymyr.pauk@gmail.com

Education:

2005 – 2009	Bc. study, Analytical Chemistry, Chemical Faculty, Uzhhorod National University. Thesis: *Ion selective electrode for determination of perchlorate*.
2009 – 2010	M.Sc. study, Analytical Chemistry, Chemical Faculty, Uzhhorod National University. Thesis: *Potentiometric sensor for determination of Chlorate (VII) ions*.
2010 – now	Ph.D. study, Analytical Chemistry, Faculty of Science, Palacký University in Olomouc. Topic: Forensic application of mass spectrometry.

Internships:

2009 – 2010	Research/Study stay, Institute of Chemistry, Faculty of Science, P. J. Šafárik University in Košice, Slovak Republic, 10 months (supervisor: prof. Yaroslav Bazeľ). Topic: Development of the new methods for the chlorates determination
2013	Research stay, Department of Chemistry, University of Washington, Seattle, WA, USA, 3 months (supervisors: prof. František Tureček, Michael Volný). Topic: Interfacing Droplets with Mass Spectrometry for Single Cell Analysis.
2014	Work stay, TSA-QC department, TEVA Czech Industries, Opava-Komárov, 3 months (supervisor: Lukáš Dvořák). Subject: Validation of analytical methods.

Projects:

2009	International Visegrad Fund, VSP 50910788 *Development of the new methods for the chlorates determination*.
2012	University Development Fund (FRVŠ), 2004/2012/G6 *Creation of new laboratory exercises utilizing non-aqueous environment for innovation of Practicals in Analaytical Chemistry*, team member.
2013	University Development Fund (FRVŠ), 1188/2013/G6 *Creation of new laboratory exercises of food analysis for innovation of Advanced Analytical Chemistry*, team member.
2014	Ministry of education, youth and sports (MŠMT), Kontakt LH14064 *Analytical tools for fast identification of new designer drugs*, team member.

Pedagogical activities:

2009 – 2010	Lectures and seminars in chemistry for students of middle school, total 8 weeks.
2011	Teaching of Practicals in Analytical Chemistry (ACH/ACC), 1 semester.
2012	Teaching of Applied Analytical Chemistry (ACH/ACHSB), 1 semester.
2013	Teaching of English for Chemists 2 (ACH/CHA2), 6 weeks.

Publications:

V. Pauk, V. Havlíček, B. Papoušková, P. Sulovský, K Lemr, Simultaneous identification of historical pigments Prussian blue and indigo in paintings by electrospray mass spectrometry, *J. Mass. Spectrom.* 48 (2013) 927–930. doi: 10.1002/jms.3228.

V. Pauk, P. Barták, K. Lemr, Characterization of natural organic colorants in historical and art objects by high-performance liquid chromatography, *J. Sep. Sci.* 37 (2014) 3393–3410. doi: 10.1002/jssc.201400650.

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See Appendix D for details.

International conferences:

Analitika RB – 2010, Minsk, Belarus, 14-15.5.2010, poster: V. Pauk., Y. Bazel – Новый перхлорат-селективный электрод с пластифицированной мембраной (A new perchlorate-selective electrode with plastificated membrane), Abstract book, 47.

53rd Hungarian Spectrochemical conference, Hajdúszoboszló, Hungary, 30.6–2.7.2010, poster: V. Pauk, Y. Bazel, J. Balogh – Development of a new perchlorate-selective electrode.

IMMS 2012, Olomouc, Czech Republic, 29.4–3.5.2012, poster: V. Pauk, B. Papoušková, P. Sulovský, K. Lemr – Identification of historical pigments Prussian Blue and Indigo by FIA/ESI-MS, *Chemica* 49S (2012) 119. Best poster prize.

4th EuCheMS Chemistry Congress, Prague, Czech Republic, 26–30.8.2012, poster: V. Pauk, B. Papoušková, P. Sulovský, K. Lemr – Mass spectrometric approach to evaluation of historical paintings by identification of Prussian blue and indigo, *Chem. Listy* 106 (2012) s1143.

Other activities:

Member of the organizing committee of Advances in Chromatography and Electrophoresis & Chiranal conference 2012 – 2014.

Contract research for companies Waters, Contipro, Novato, Atotech, Synthesia and TEVA Czech Industries.

Consultation of master thesis: Veronika Žihlová, Lucie Pušová (successfully finished).

Reviewer of the bachelor and master theses.

APPENDICES

Appendix A. Identification of Prussian blue and indigo by FIA/ESI-MS

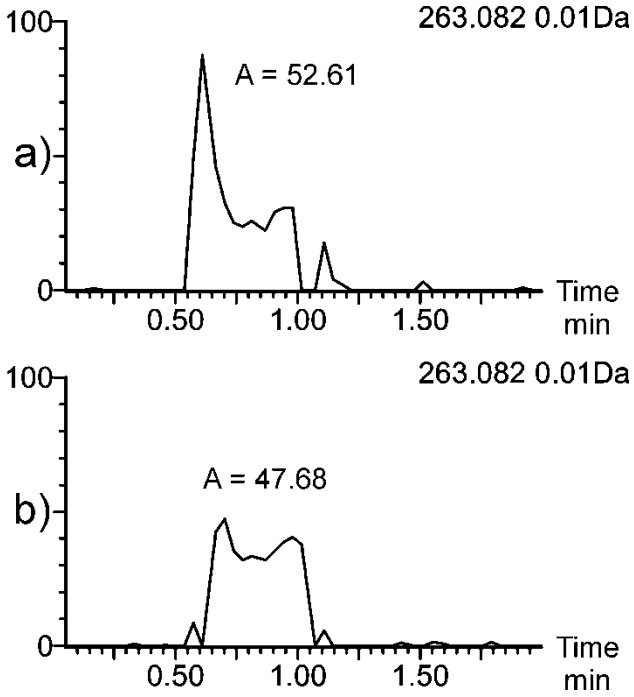

Figure A1. FIA mass-chronograms of **a)** 50 ng/ml freshly reduced indigo, **b)** the same solution after one week storage. Integrated peak areas are shown.

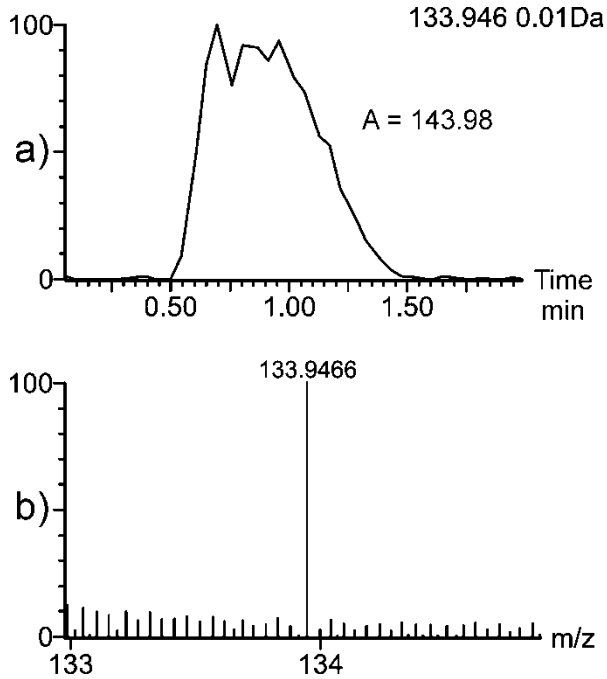

Figure A2. FIA mass chronogram, **a)** and MS spectrum, **b)** showing interference in blank with sodium dithionite. Integrated peak area is shown.

Appendix B. Differentiation of plant gum binders by SFC/MS

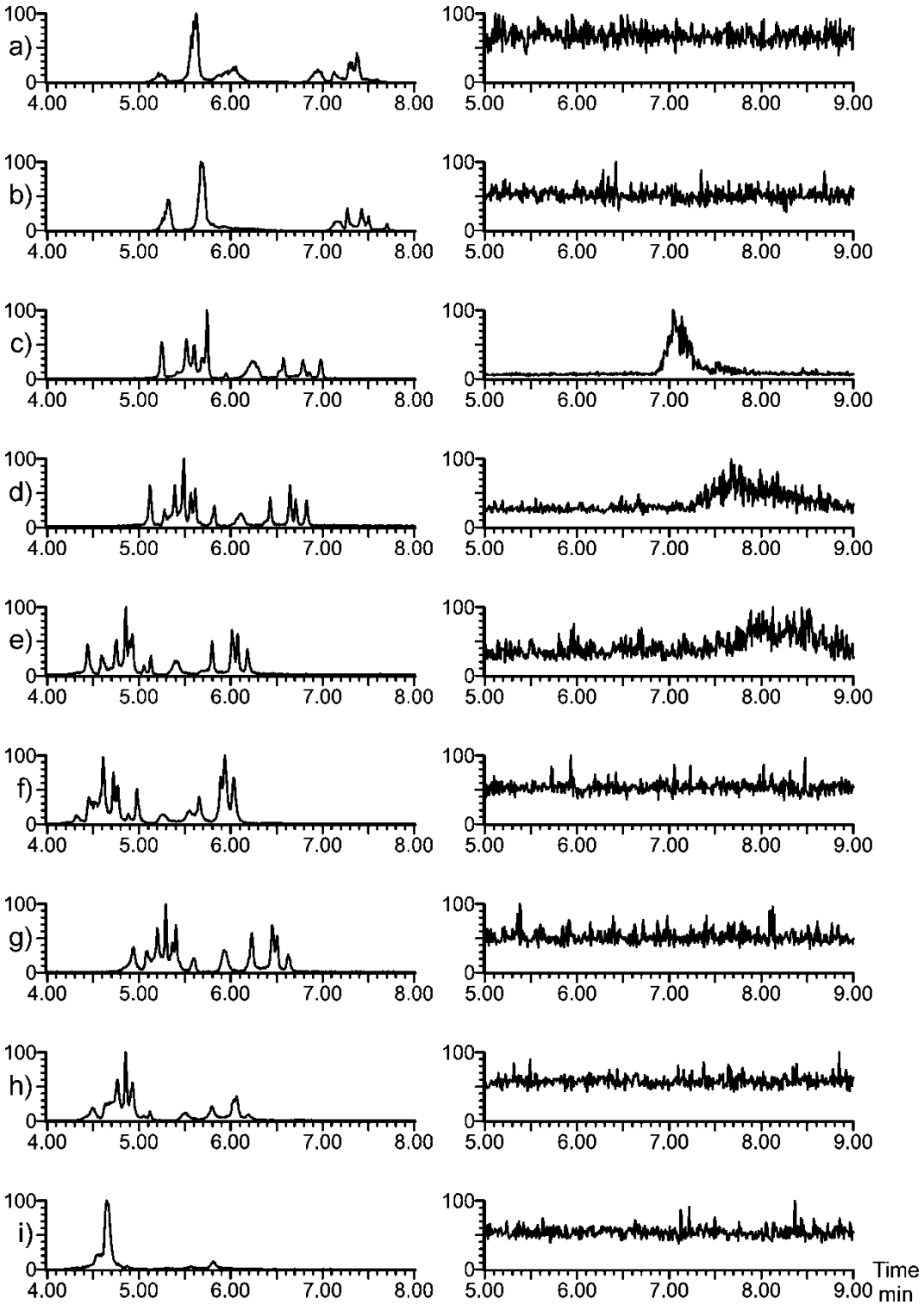

Figure B1. Evaluation of additives in methanol on BEH: **a)** 5% water, 20 mM NH_4OH; **b)** 5% water, 20 mM $HCOONH_4$; **c)** 5% water, 4 % HCOOH; **d)** 5% water, 2 % HCOOH; **e)** 2% water, 1% HCOOH; **f)** 1% HCOOH; **g)** 5% water; **h)** 2% water; **i)** pure MeOH. Left: positive MS mode, right: negative mode.

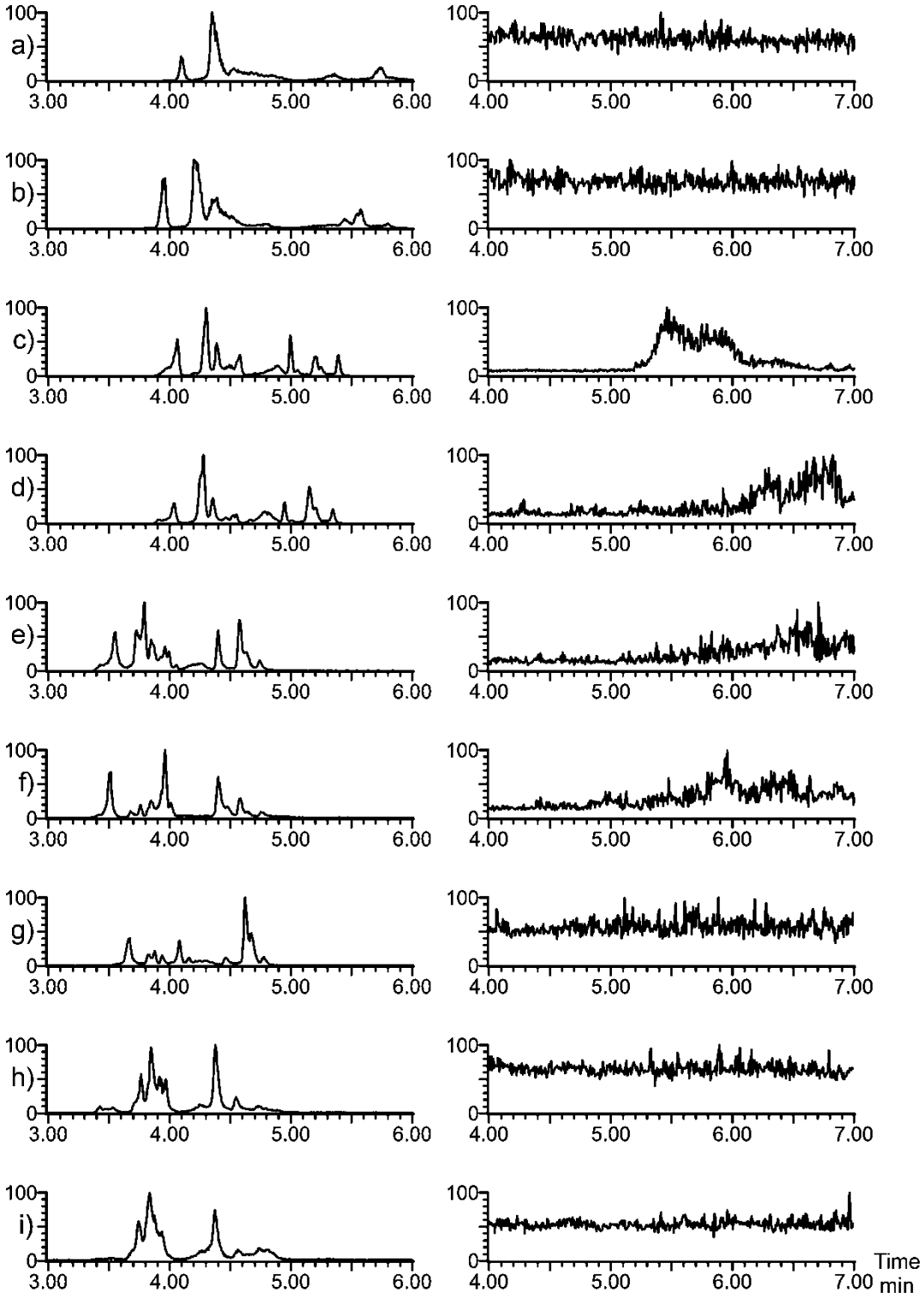

Figure B2. Evaluation of additives in methanol on C18SB: **a)** 5% water, 20 mM NH_4OH; **b)** 5% water, 20 mM $HCOONH_4$; **c)** 5% water, 4% HCOOH; **d)** 5% water, 2% HCOOH; **e)** 2% water, 1% HCOOH; **f)** 1% HCOOH; **g)** 5% water; **h)** 2% water; **i)** pure MeOH. Left: positive MS mode, right: negative mode.

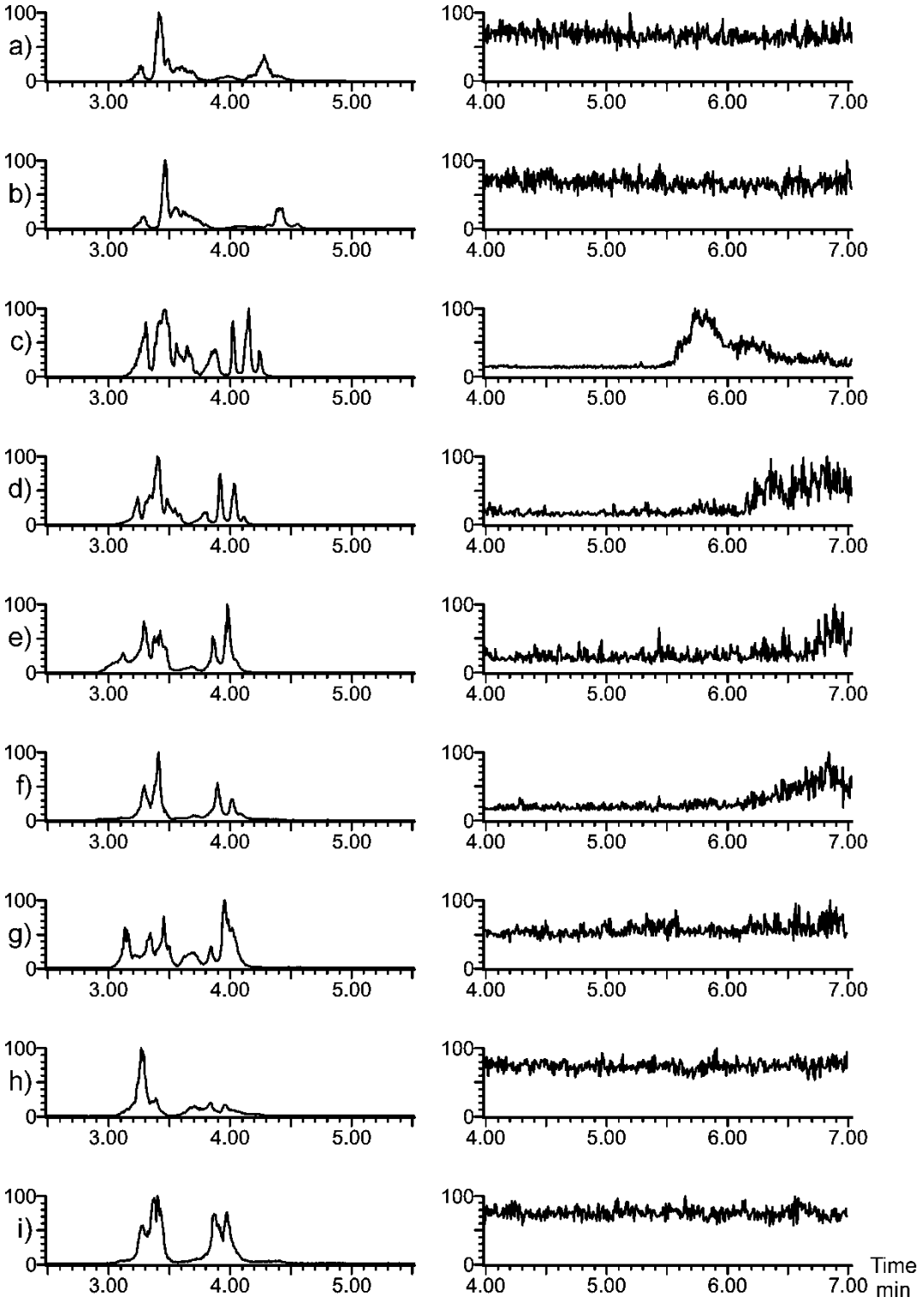

Figure B3. Evaluation of additives in methanol on Fluoro-Phenyl: **a)** 5% water, 20 mM NH_4OH; **b)** 5% water, 20 mM $HCOONH_4$; **c)** 5% water, 4% HCOOH; **d)** 5% water, 2% HCOOH; **e)** 2% water, 1% HCOOH; **f)** 1% HCOOH; **g)** 5% water; **h)** 2% water; **i)** pure MeOH. Left: positive MS mode, right: negative mode.

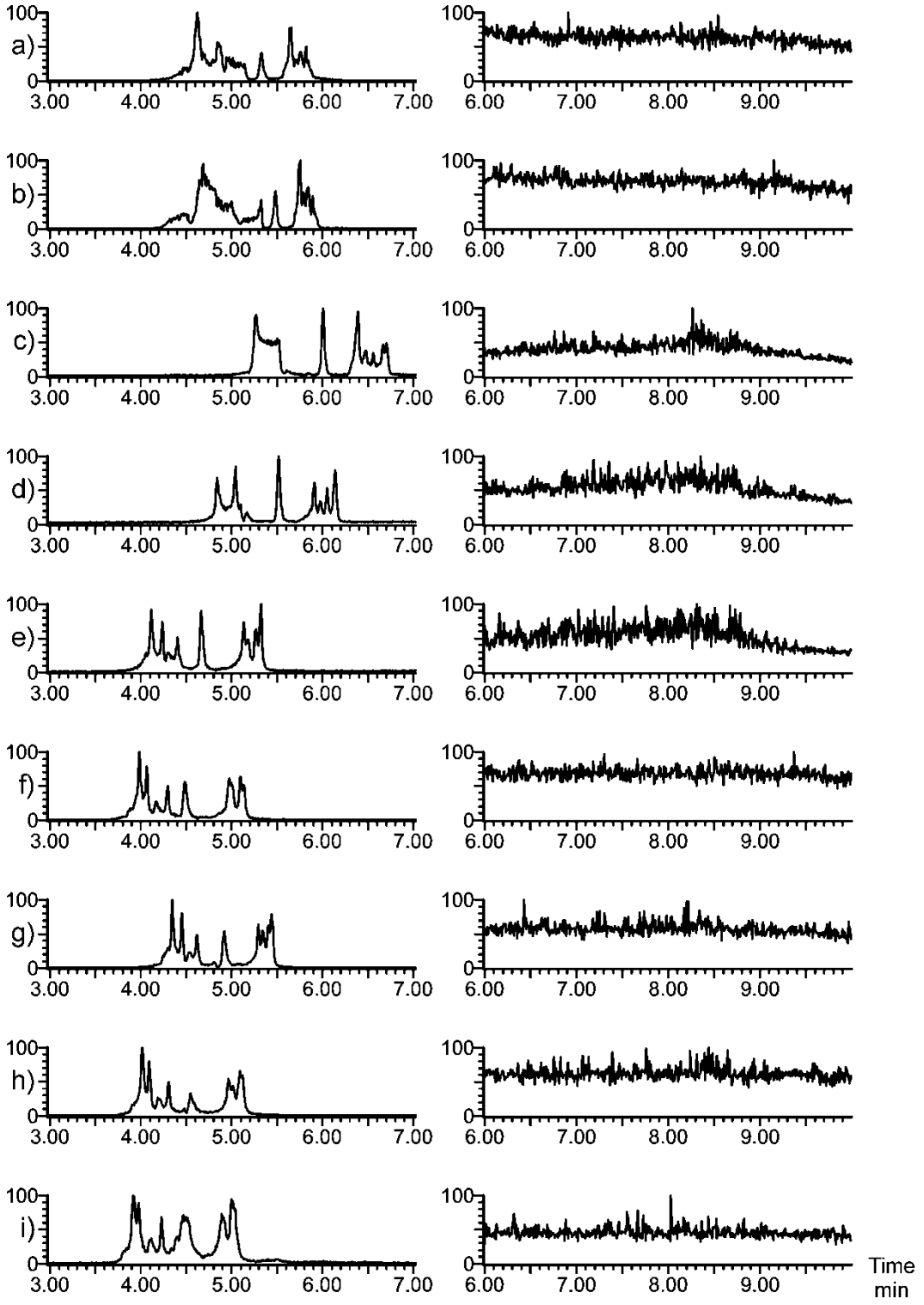

Figure B4. Evaluation of additives in methanol on 2-EP: **a)** 5% water, 20 mM NH_4OH; **b)** 5% water, 20 mM $HCOONH_4$; **c)** 5% water, 4% HCOOH; **d)** 5% water, 2% HCOOH; **e)** 2% water, 1% HCOOH; **f)** 1% HCOOH; **g)** 5% water; **h)** 2% water; **i)** pure MeOH. Left: positive MS mode, right: negative mode.

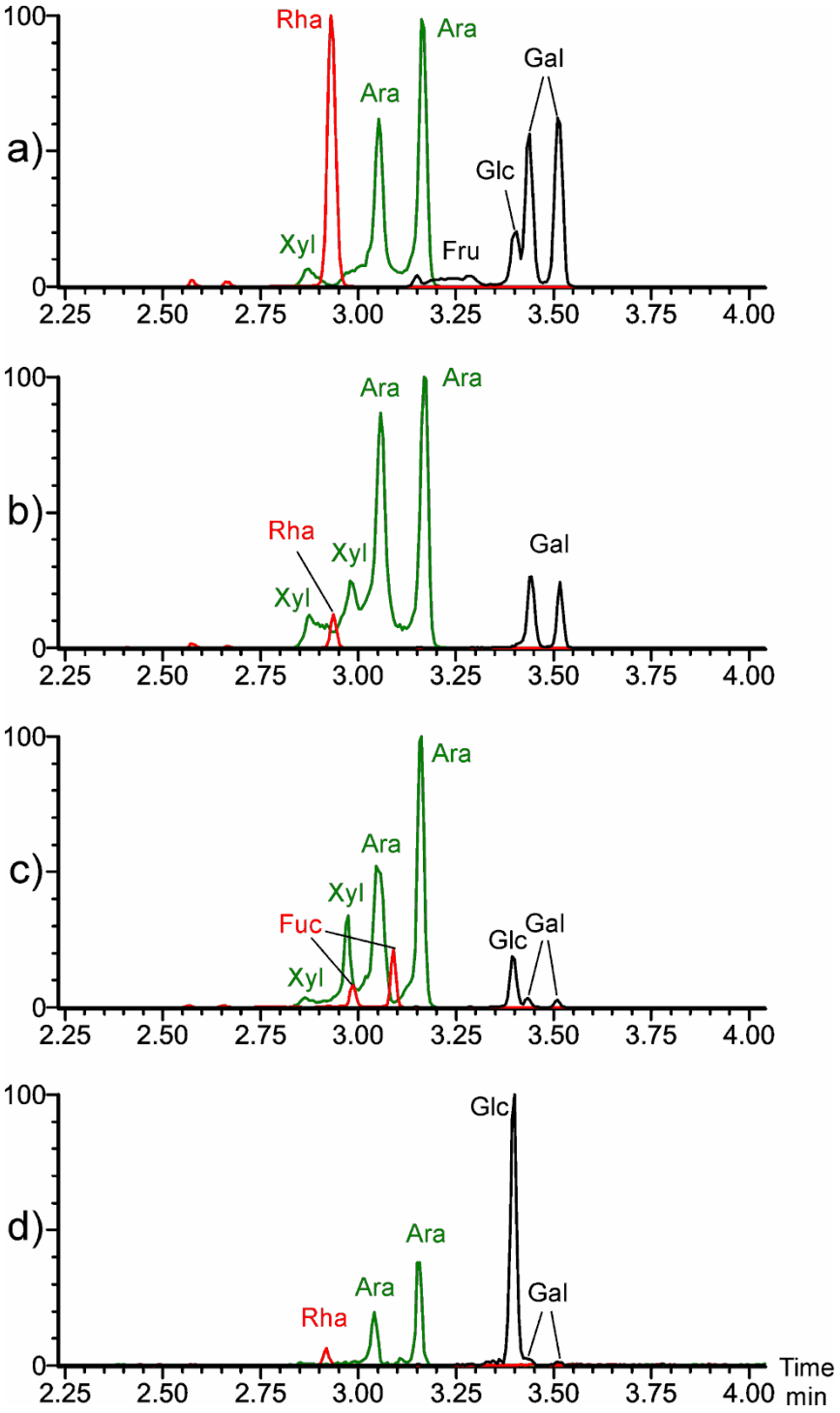

Figure B5. Representative chromatograms of plant gums and aquarelle sample on C18SB: **a)** gum Arabic, **b)** cherry gum, **c)** gum tragacanth, **d)** ultramarine.

Appendix C. Development of SFC/MS method for analysis of polar designer drugs

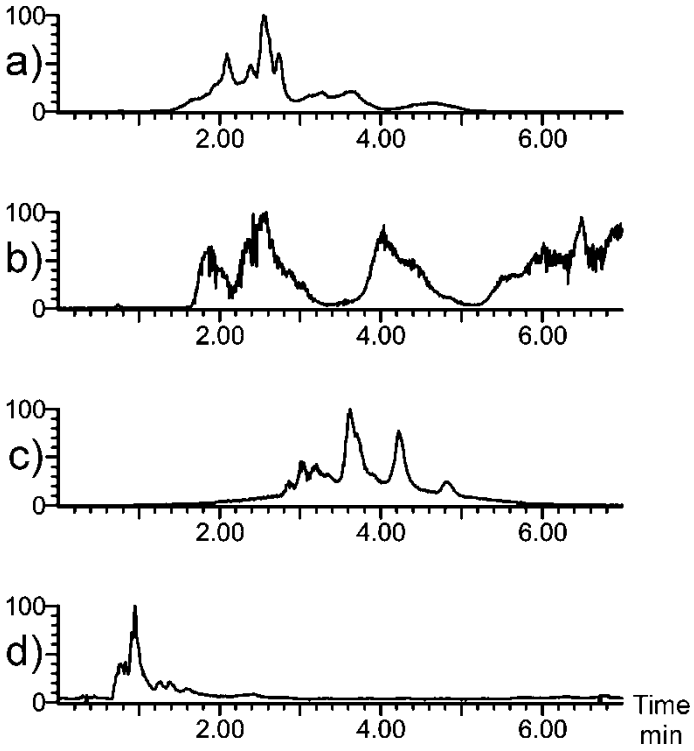

Figure C1. Methanol, 50 °C, **a)** BEH; **b)** Fluoro-Phenyl; **c)** C18SB; **d)** 2-EP.

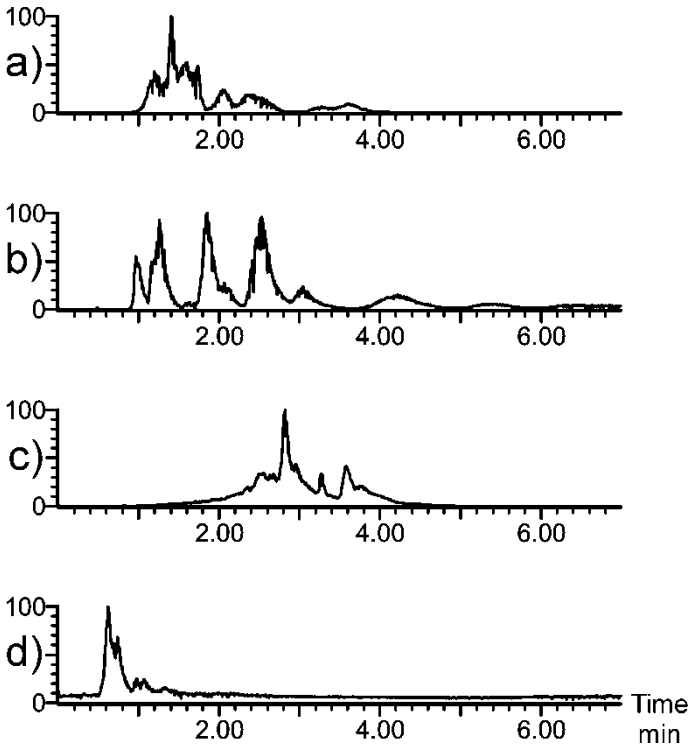

Figure C2. 2% water in methanol, 50 °C, **a)** BEH; **b)** Fluoro-Phenyl; **c)** C18SB; **d)** 2-EP.

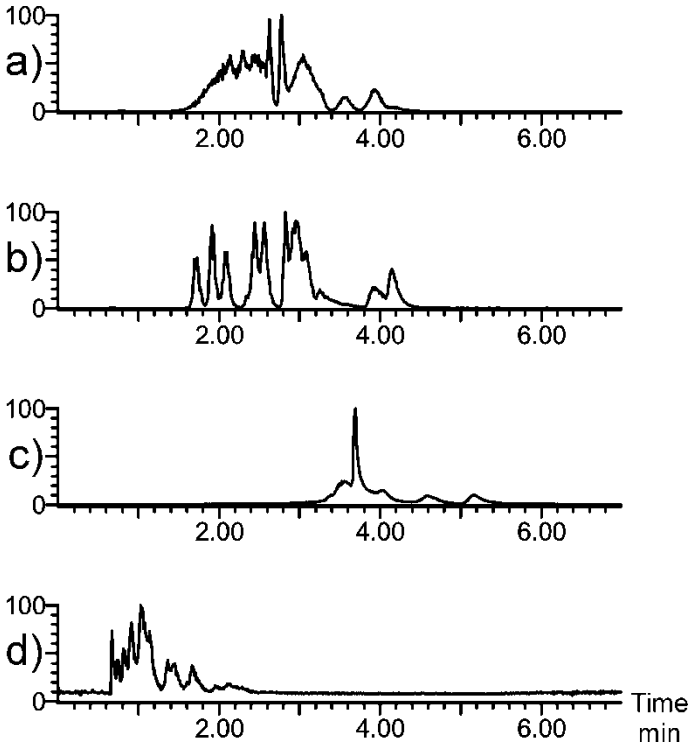

Figure C3. 20 mM HCOOH in methanol, 50 °C, **a)** BEH; **b)** Fluoro-Phenyl; **c)** C18SB; **d)** 2-EP.

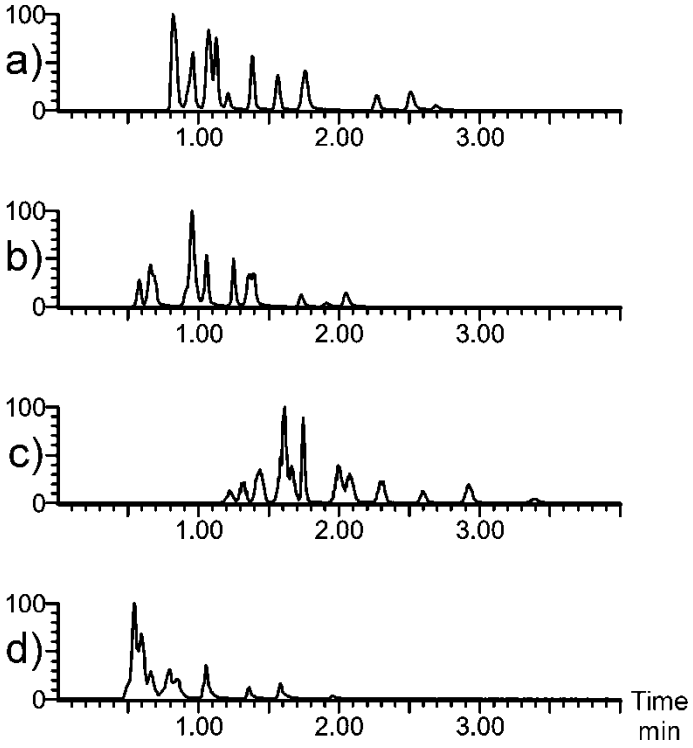

Figure C4. 20 mM NH_4OH in methanol, 50 °C, **a)** BEH; **b)** Fluoro-Phenyl; **c)** C18SB; **d)** 2-EP.

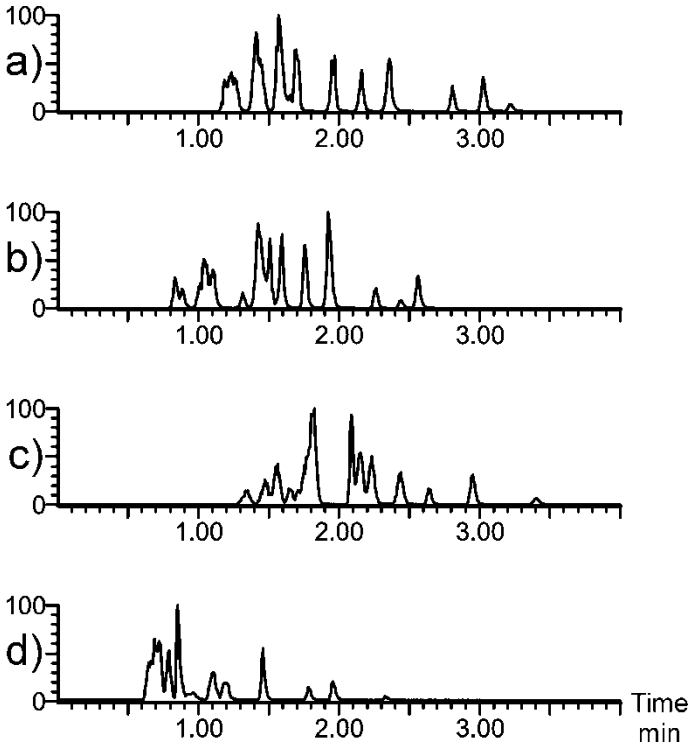

Figure C5. 20 mM $HCOONH_4$ in methanol, 50 °C, **a)** BEH; **b)** Fluoro-Phenyl; **c)** C18SB; **d)** 2-EP.

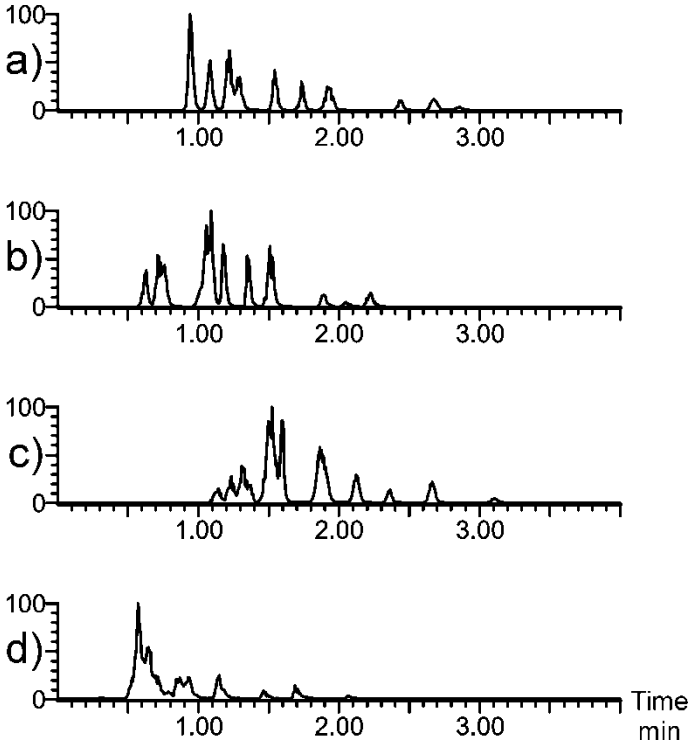

Figure C6. 20 mM CH_3COONH_4 in methanol, 50 °C, **a)** BEH; **b)** Fluoro-Phenyl; **c)** C18SB; **d)** 2-EP.

Table C1. Properties* of investigated cathinones and phenylethylamines.

#	Substance	Molecular formula	MI mass	pK_a	log *P*	PSA, A^2	A	B	S	E	V
1	cathinone	$C_9H_{11}NO$	149.08406	7.97	0.916	43.1	0.21	1	1.44	1	1.2546
2	buphedrone	$C_{11}H_{15}NO$	177.11536	7.14	0.862	29.1	0.13	0.9	1.3	0.95	1.5364
3	3-MMC	$C_{11}H_{15}NO$	177.11536	7.84	0.469	29.1	0.13	0.9	1.24	0.97	1.5364
4	MDA	$C_{10}H_{13}NO_2$	179.09462	9.94	1.637	44.5	0.21	1.08	1.23	1.11	1.3886
5	3-FMC	$C_{10}H_{12}NOF$	181.09028	7.14	0.598	29.1	0.13	0.9	1.26	0.86	1.4131
6	flephedrone	$C_{10}H_{12}NOF$	181.09028	7.24	0.833	29.1	0.13	0.9	1.26	0.86	1.4131
7	2C-H	$C_{10}H_{15}NO_2$	181.11027	9.72	1.4	44.5	0.21	1.08	1.18	0.91	1.4972
8	4-MEC	$C_{12}H_{17}NO$	191.13101	7.43	1.186	29.1	0.13	0.9	1.24	0.97	1.6773
9	BDB	$C_{11}H_{15}NO_2$	193.11027	10.0	2.146	44.5	0.21	1.09	1.23	1.1	1.5295
10	methedrone	$C_{11}H_{15}NO_2$	193.11027	7.48	0.528	38.3	0.13	1.11	1.39	1.01	1.5951
11	methylone	$C_{11}H_{13}NO_3$	207.08953	7.74	-0.396	47.6	0.13	1.29	1.57	1.27	1.5452
12	ethylone	$C_{12}H_{15}NO_3$	221.10518	7.75	0.114	47.6	0.13	1.29	1.58	1.27	1.6861
13	butylone	$C_{12}H_{15}NO_3$	221.10518	7.74	0.114	47.6	0.13	1.29	1.58	1.27	1.6861
14	2C-B	$C_{10}H_{14}NO_2Br$	259.02078 261.01873	9.37	2.25	44.5	0.21	1.01	1.33	1.2	1.6722
15	naphyrone	$C_{19}H_{23}NO$	281.17796	8.44	4.363	20.3	0	1	1.73	1.89	2.3604

*Dissociation constant pK_a, partition coefficient log *P* and Polar surface area, PSA, taken from https://scifinder.cas.org. Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02, for 25 °C. Abraham solvation parameters were calculated using Absolv (v5.0.0.184) available at https://ilab.acdlabs.com/iLab2/ (accessed 18.09.2014).

Table C2. Evaluation of BEH.

Additive	Temp., °C	Isomers			All compounds					
		R>1	1>*R*>0.3	*R*<0.3	*R*>1	1>*R*>0.3	*R*<0.3	Gauss.	Time, min	$W_{1/2}$, s
NH_4OH, 5 mM	40	2	2	0	6	8	1	12	3.39	4.91
NH_4OH, 10 mM	40	**4**	0	0	9	4	2	12	3.35	3.66
NH_4OH, 20 mM	**40**	**4**	**0**	**0**	**11**	**2**	**2**	**15**	**2.87**	**2.51**
	50	3	1	0	10	4	1	12	2.86	2.22
	60	3	0	1	11	3	1	12	2.79	2.22
$HCOONH_4$, 5 mM	40	2	2	0	6	8	1	11	3.57	5.06
$HCOONH_4$, 10 mM	40	**4**	0	0	8	6	1	12	3.59	3.98
$HCOONH_4$, 20 mM	**40**	**4**	**0**	**0**	**11**	**2**	**2**	**10**	**3.25**	**2.61**
	50	**4**	0	0	10	5	0	10	3.40	2.38
	60	**4**	0	0	10	3	2	12	3.38	2.56
CH_3COONH_4	**40**	**4**	**0**	**0**	**10**	**3**	**2**	**14**	**3.01**	**2.72**
	50	**4**	0	0	10	2	3	15	2.99	2.42
	60	3	0	1	8	6	1	14	3.07	2.60
HCOOH	40	2	0	2	4	5	6	1	4.61	11.02
	50	0	2	2	5	6	4	0	4.58	11.32
	60	2	0	2	5	8	2	2	4.52	7.12

Table C3. Evaluation of Fluoro-Phenyl.

Additive	Temp., °C	Isomers			All compounds					
		R>1	1>*R*>0.3	*R*<0.3	*R*>1	1>*R*>0.3	*R*<0.3	Gauss.	Time, min	$W_{1/2}$, s
NH_4OH, 5 mM	60	3	1	0	9	4	2	9	2.97	3.14
NH_4OH, 10 mM	60	3	0	1	9	4	2	11	2.62	2.51
NH_4OH, 20 mM	40	3	1	0	9	3	3	15	2.19	2.47
	50	3	1	0	9	4	2	13	2.24	2.12
	60	**4**	**0**	**0**	**10**	**4**	**1**	**11**	**2.36**	**1.99**
$HCOONH_4$, 5 mM	60	3	1	0	9	6	0	12	3.01	3.08
$HCOONH_4$, 10 mM	60	3	1	0	9	5	1	10	2.78	2.78
$HCOONH_4$, 20 mM	40	3	1	0	10	4	1	10	2.55	2.76
	50	3	0	1	10	3	2	12	2.67	2.75
	60	3	1	0	10	4	1	**12**	2.78	2.05
$HCOONH_4$, 20 mM; 2% H_2O	**60**	**4**	**0**	**0**	**10**	**4**	**1**	**10**	**2.62**	**2.34**
CH_3COONH_4	40	3	0	1	9	3	3	15	2.28	2.68
	50	3	0	1	9	4	2	10	2.37	2.50
	60	3	0	1	**10**	2	3	9	2.48	2.10
HCOOH	40	**4**	0	0	7	5	3	4	5.12	7.86
	50	3	1	0	6	8	1	5	4.52	6.44
	60	3	0	1	9	5	1	3	4.76	5.97

Table C4. Evaluation of C18SB.

Additive	Temp., °C	Isomers			All compounds					
		R>1	1>*R*>0.3	*R*<0.3	*R*>1	1>*R*>0.3	*R*<0.3	Gauss.	Time, min	$W_{1/2}$, s
NH_4OH, 20 mM	40	3	0	1	9	3	3	10	3.56	3.35
	50	3	0	1	**10**	3	2	10	3.53	2.92
	60	3	0	1	8	3	4	13	3.47	2.59
$HCOONH_4$, 20 mM	40	3	0	1	8	3	4	8	3.47	3.31
	50	3	0	1	8	6	1	11	3.51	2.66
	60	**3**	**0**	**1**	**12**	**1**	**2**	**12**	**3.53**	**2.27**
$HCOONH_4$, 20 mM; 2% H_2O	**60**	**3**	**1**	**0**	**12**	**2**	**1**	**13**	**3.67**	**2.30**
CH_3COONH_4	40	3	0	1	9	3	3	9	3.20	3.38
	50	3	0	1	9	**4**	2	15	3.22	2.81
	60	3	0	1	8	5	2	14	3.28	2.59
HCOOH	40	1	0	3	4	2	8	1	6.38	6.63
	50	3	0	1	4	5	6	1	6.56	8.44
	60	2	1	1	3	9	3	2	6.99	10.96

Table C5. Evaluation of 2-EP.

Additive	Temp., °C	Isomers compounds			All compounds					
		R>1	1>*R*>0.3	*R*<0.3	*R*>1	1>*R*>0.3	*R*<0.3	Gauss.	Time, min	$W_{1/2}$, s
NH_4OH, 20 mM	**40**	**2**	**1**	**1**	**6**	**5**	**4**	**10**	**2.08**	**2.29**
	50	**2**	**1**	**1**	**6**	**7**	**2**	**9**	**2.11**	**2.35**
	60	**2**	**1**	**1**	**6**	**7**	**2**	**8**	**2.21**	**2.26**
$HCOONH_4$, 20 mM	40	2	0	2	9	3	3	11	2.38	2.50
	50	2	0	2	**9**	**4**	**2**	**11**	2.45	2.28
	60	2	0	2	7	6	2	10	2.55	2.46
CH_3COONH_4	40	2	0	2	6	5	4	8	2.23	2.95
	50	2	0	2	8	4	3	8	2.26	2.68
	60	**2**	**1**	**1**	**6**	**7**	**2**	**6**	**2.38**	**2.38**
HCOOH	40	0	3	1	2	9	4	0	3.30	11.37
	50	0	2	2	2	9	4	0	3.38	10.44

Shaded rows and rows in bold represent the best and second-best results, respectively, for a chosen column. Single values in bold represent the determinative criteria for a chosen additive.

Table C6. Resolution of isomers on BEH.

Additive	Temp., °C	ethylone	3-FMC	buphedrone	BDB
		butylone	flephedrone	3-MMC	methedrone
NH_4OH 5 mM	40	0.88	1.43	1.77	0.80
NH_4OH 10 mM	**40**	**1.17**	**2.00**	**2.17**	**1.53**
NH_4OH 20 mM	**40**	**1.11**	**2.00**	**3.03**	**2.15**
	50	0.75	1.82	3.74	2.76
	60	0.00	1.56	3.63	2.58
$HCOONH_4$ 5 mM	40	0.80	2.01	1.39	0.91
$HCOONH_4$ 10 mM	**40**	**1.07**	**1.70**	**2.85**	**1.41**
$HCOONH_4$ 20 mM	**40**	**1.20**	**2.22**	**3.83**	**2.32**
	50	**1.25**	**3.16**	**5.00**	**3.11**
	60	0.98	2.08	3.99	2.85
CH_3COONH_4 20 mM	**40**	**1.29**	**2.44**	**3.97**	**2.13**
	50	**1.19**	**2.19**	**3.77**	**2.61**
	60	0.00	1.88	3.57	2.08
HCOOH 20 mM	40	0.00	0.01	1.28	1.41
	50	0.00	0.03	0.72	0.46
	60	0.00	0.96	1.61	0.17

Table C7. Resolution of isomers on Fluoro-Phenyl.

Additive	Temp., °C	ethylone	3-FMC	buphedrone	BDB
		butylone	flephedrone	3-MMC	methedrone
NH_4OH 5 mM	60	1.07	2.28	4.31	0.62
NH_4OH 10 mM	60	1.55	1.77	4.48	0.00
NH_4OH 20 mM	40	1.68	1.35	3.45	0.44
	50	1.52	1.20	4.25	0.52
	60	**1.42**	**1.06**	**4.45**	**1.07**
$HCOONH_4$ 5 mM	60	1.61	1.95	4.11	0.95
$HCOONH_4$ 10 mM	60	1.72	1.14	4.94	0.95
$HCOONH_4$ 20 mM	40	1.84	2.45	3.01	0.49
	50	1.88	1.04	4.63	0.21
	60	2.09	2.22	5.07	0.67
$HCOONH_4$ 20 mM, 2% H_2O	**60**	**2.33**	**1.17**	**4.90**	**1.17**
CH_3COONH_4 20 mM	40	1.78	1.58	3.31	0.23
	50	2.05	1.57	4.66	0.00
	60	1.79	1.18	6.14	0.10
HCOOH 20 mM	**40**	**1.51**	**1.09**	**1.58**	**1.04**
	50	2.21	1.45	3.57	0.75
	60	2.93	2.06	4.77	0.14

Table C8. Resolution of isomers on C18SB.

Additive	Temp., °C	ethylone	3-FMC	buphedrone	BDB
		butylone	flephedrone	3-MMC	methedrone
NH_4OH 20 mM	40	0.00	3.58	2.56	2.27
	50	0.00	2.15	4.40	2.24
	60	0.00	2.13	4.41	2.72
$HCOONH_4$ 20 mM	40	0.00	2.65	2.98	2.46
	50	0.00	2.42	4.73	2.48
	60	0.00	3.00	5.06	1.88
$HCOONH_4$ 20 mM, 2% H_2O	**60**	**0.56**	**3.17**	**5.16**	**2.23**
CH_3COONH_4 20 mM	40	0.00	3.02	3.50	2.55
	50	0.00	2.36	4.46	2.38
	60	0.00	2.24	3.77	2.48
HCOOH 20 mM	40	0.00	0.00	0.23	4.49
	50	0.00	1.03	1.68	6.03
	60	0.00	0.94	1.37	2.97

Table C9. Resolution of isomers on 2-EP.

Additive	Temp., °C	ethylone	3-FMC	buphedrone	BDB
		butylone	flephedrone	3-MMC	methedrone
NH_4OH 20 mM	**40**	**0.81**	**0.00**	**0.98**	**2.91**
	50	0.67	0.00	0.99	2.69
	60	0.76	0.00	1.01	2.71
$HCOONH_4$ 20 mM	40	0.00	0.09	2.02	3.45
	50	0.00	0.00	2.47	3.50
	60	0.00	0.00	2.47	3.00
CH_3COONH_4 20 mM	40	0.00	0.15	0.98	2.88
	50	0.00	0.00	1.07	3.24
	60	0.70	0.08	1.04	2.73
HCOOH 20 mM	40	0.68	0.02	0.53	0.57
	50	0.00	0.05	0.71	0.81

Table C10. Retention factor k of investigated compounds*.

#	Substance	k, BEH,		k, F-Ph		k, C18SB		k, 2-EP	
		AmH	AmF	AmH	AmF	AmH	AmF	AmH	AmF
1	cathinone	3.37	4.96	2.47	3.87	5.29	5.65	1.76	2.53
2	buphedrone	2.01	3.55	1.55	3.10	4.13	4.74	0.91	1.35
3	3-MMC	2.84	4.67	2.51	4.28	5.47	6.10	1.13	1.90
4	MDA	7.19	9.14	5.40	7.34	9.10	9.27	4.00	5.56
5	3-FMC	2.03	3.33	1.14	2.17	3.76	4.21	1.02	1.54
6	flephedrone	2.47	4.09	1.42	2.84	4.53	5.07	1.02	1.54
7	2C-H	8.06	9.90	6.56	8.46	10.36	10.48	4.81	6.21
8	4-MEC	2.36	4.27	2.36	4.24	4.67	5.42	0.84	1.43
9	BDB	5.35	7.52	4.02	6.14	7.06	7.68	2.86	4.37
10	methedrone	4.65	6.80	4.15	6.08	7.95	8.47	2.13	3.38
11	methylone	4.00	6.08	3.61	5.49	6.77	7.37	1.94	3.04
12	ethylone	3.08	5.14	2.91	4.89	5.23	6.04	1.24	2.13
13	butylone	2.94	4.74	2.60	4.39	5.23	5.83	1.43	2.13
14	2C-B	8.71	10.62	6.05	8.00	12.17	12.23	6.21	7.57
15	naphyrone	1.94	4.16	2.52	4.57	5.80	7.13	0.99	1.68

*Retention factors are given for 50 ºC.

Appendix D. Publications related to the thesis

V. Pauk, V. Havlíček, B. Papoušková, P. Sulovský, K. Lemr, Simultaneous identification of historical pigments Prussian blue and indigo in paintings by electrospray mass spectrometry, *J. Mass. Spectrom.* 48 (2013) 927–930. DOI: 10.1002/jms.3228.

V. Pauk, P. Barták, K. Lemr, Characterization of natural organic colorants in historical and art objects by high-performance liquid chromatography, *J Sep Sci.* 37 (2014) 3393–410. DOI: 10.1002/jssc.201400650.

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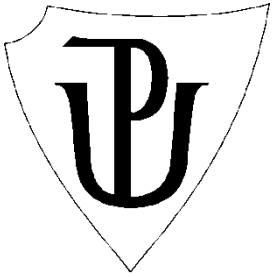
Faculty of Science
Department of Analytical Chemistry



FORENSIC APPLICATIONS OF MASS SPECTROMETRY

SUMMARY
OF THE DOCTORAL THESIS

Author: Volodymyr Pauk

Field of study: Analytical Chemistry

Supervisor: prof. RNDr. Karel Lemr, Ph.D.

Olomouc 2015

ABSTRACT

The Ph.D. thesis is devoted to development of new MS-based methods useful for identification of specific substances in objects within the scope of forensic science. The work is divided into three sections.

The first one deals with the identification of historical pigments indigo and Prussian blue in oil paintings. Analysis is based on simple chemical reactions, hydrolysis of Prussian blue and reduction of indigotine in alkaline environment, leading to efficient dissolving and sensitive detection of the pigments. The developed FIA/ESI-MS method is fast and makes possible identification of both inorganic and organic components without chromatographic separation. The limits of detection are at picogram levels. Potential of the developed method was proven in analysis of blue samples from two oil paintings (20^{th} century) and a microsample from the painting of 'Crucifixion', St. Šebestián church on St. Hill, Mikulov.

The second section deals with differentiation of plant gums used as binders in historical and art objects. Plant-derived polysaccharides are decomposed prior the analysis by microwave-assisted hydrolysis. Hydrolysates containing monosaccharides in different ratios specific to each type of plant gum are analyzed by means of supercritical fluid chromatography hyphenated to mass spectrometry. Subsequently, chromatographic data is subjected to PCA analysis which reveals differences in composition of plant gums. Samples of high-grade aquarelle paints and one archaeological sample were analyzed and compared with profiles of the three most widespread plant glums (gum Arabic, cherry gum and gum tragacanth).

The third section describes development of a new method for analysis of modern synthetic drugs of abuse, so-called "new designer drugs", belonging to classes of cathinones and phenylethylamines. The analysis is based on supercritical fluid chromatography with mass spectrometric detection. Efficient separation of 4 isomeric pairs and most of remaining analytes (15 compounds in total) was achieved in less than 3 min on BEH (silica) and Fluoro-phenyl stationary phases, with appropriate mobile phase modifiers. Electrospray ionization with triple quadrupole analyzer in SRM mode provided an additional dimension for differentiation and sensitive detection of all investigated substances.

ABSTRAKT

Disertační práce se zabývá aplikacemi hmotnostní spektrometrie ve forenzní vědě. Práce je rozdělena do tří oddílů.

První oddíl pojednává o identifikaci historických pigmentů indiga a Pruské modři v olejových malbách. Analýza je založena na jednoduchých chemických reakcích (hydrolýza Pruské modři a redukce indigotinu v alkalickém prostředí) umožňujících rozpouštění pigmentů a jejich citlivou detekci. Vyvinutá FIA/ESI-MS metoda je rychlá a umožňuje identifikaci obou látek bez chromatografické separace. Meze detekce jsou na úrovni pikogramů. Možnosti této metody byly prokázány při analýze vzorků modré barvy ze dvou olejových maleb (počátek 20. století) a mikrovzorku z obrazu „Ukřižování“, kostel Sv. Šebestiána na Sv. Kopečku v Mikulově.

Druhý oddíl je zaměřen na rozpoznání rostlinných gum používaných jako pojiva v historických a uměleckých předmětech. Rostlinné polysacharidy se před analýzou hydrolyzují pomocí mikrovlnného záření. Hydrolyzáty obsahující monosacharidy v různých poměrech, specifických pro každý typ gumy, jsou analyzovány pomocí superkritické fluidní chromatografie s hmotnostní detekcí. Následně jsou experimentální data zpracována metodou hlavních komponent (PCA), která je schopna odhalit rozdíly ve složení jednotlivých pryskyřic. Vzorky akvarelů vysoké kvality a jeden archeologický vzorek byly analyzovány a srovnány s profily třech nejčastěji používaných přírodních gum (Arabská guma, třešňová guma a tragant).

Třetí oddíl popisuje vývoj metody pro analýzu nových syntetických drog, tzv. “new designer drugs”, patřících do skupin katinonů a fenylethylaminů. Analýza je založena na separaci pomocí superkritické fluidní chromatografie s hmotnostní detekcí. Použití stacionárních fází BEH (silikagel) a Fluoro-phenyl s vhodnými modifikátory v mobilní fázi umožňuje separaci většiny z 15 studovaných návykových látek, především 4 párů isomerů, méně než za 3 min. Ionizace elektrosprejem ve spojení s trojitým kvadrupólem v SRM módu poskytuje další dimenzi pro rozlišení a citlivou detekci všech sledovaných látek.

TABLE OF CONTENTS

1 INTRODUCTION

Forensic science is an interdisciplinary field that deals not only with controlled substances, arson investigation or detection of explosives but also answers questions of authenticity, provenance and age of various valuable objects. Investigation of forgeries, fakes and copies of written documents, jewelry, historical and artistic objects is an important research area within the framework of forensic science. Powerful, sensitive and selective analytical techniques which offer all necessary information are required to address the forensic challenges.

Mass spectrometry is one of such tools that fulfill imposed requirements. It is the most versatile detection technique which provides structural information and identification of unknowns. There is a huge bunch of existing forensic protocols utilizing combination of MS and GC, LC or CE. Some of these procedures were established many years ago and are subjected to restrictions of outdated techniques. Since new instruments with higher resolution, sensitivity and efficiency are emerging, one can benefit from data-rich results, reduction of analysis time and lower sample amount.

Supercritical fluid chromatography (SFC) has gained much interest in recent years. Since its discovery SFC instrumentation has undergone a number of improvements and now it can compete with ultra-high performance liquid chromatography (UHPLC) in terms of performance. Its range of potential analytes can be significantly extended by use of various mobile phase modifiers and additives making it complementary to reversed-phase LC or even hydrophilic interaction liquid chromatography (HILIC). It is useful for resolution of isomers that cannot be unambiguously distinguished by MS.

Provided with such a wide spectrum of instruments, development of analytical methods is the priority task of an analytical chemist. Therefore, we focused our efforts on the development of new analytical protocols utilizing advantages of the mentioned techniques and instrumentation. We successfully used ESI as one of the most versatile ionization sources, high-resolution Q-TOF instrument for identification of pigments in complex oil painting matrix and a triple quadrupole analyzer for analysis of saccharides in binding medium and new designer drugs (NDDs). In the last two applications UHPSFC system allowed fast separation of isomeric compounds.

2 THEORETICAL PART

2.1 Identification of Prussian blue and indigo by FIA/ESI-MS

2.1.1 Analysis of historical pigments and dyes

Identification of historical colorants provides crucial information for preservation and restoration of art objects. The analytical data gives useful clues on the painting technique of an artist as well as on household activity and the culture of a certain historical period. Since specific colorants were used during different times or in particular geographic locations, their identification assists in evaluation of object authenticity, dating or localizing the provenance of artworks and historical artifacts.

The available amount of a sample from an art or historical object is often extremely small, but information about main and minor components, corresponding degradation products as well as quantitative ratio of substances can be required to reach a final conclusion. Various spectroscopic techniques, such as UV/Vis, IR, Raman, fluorescence spectroscopy, NMR, XRF, are potentially suitable for analysis of art objects, but each has some serious limitations [1,2]. On the contrary, combination of separation techniques and MS is sufficiently sensitive and selective, provides structural information and allows identification of unknown compounds [3]. HPLC/MS remains the method of choice for analysis of natural dyes [2]. The disadvantage is that laborious sample treatment is often necessary.

Direct MS methods, such as direct analysis in real time (DART) [4], laser desorption ionization (LDI) [5] and secondary ion mass spectrometry (SIMS) [6-8], do not require chromatographic separation and are useful for fast analysis of insoluble pigments or when confirmation of presence of a certain colorant is needed. Despite the advantages of the mentioned techniques they are not suitable for quantitative analysis and require dedicated instrumentation, additional maintenance and skilled operators.

2.1.2 Identification of insoluble blue pigments

Indigo and Prussian blue (PB) belong to important blue colorants and their distinguishing is useful for dating of artworks. Indigo has been produced from plant material (woad, *Isatis tinctoria* L. and indigo-plant, *Indigofera tinctoria* L.) since ancient times and was synthesized at the end of the 19th

century. Due to its high lightfastness it was used not only as a textile dye but as a pigment in medieval paintings, illuminations, sculptures and frescos as well [9,10]. PB is a synthetic pigment obtained for the first time at the beginning of the 18th century in Berlin [11]. It has become used in watercolors and substituted indigo in oil paintings [12]. Discrimination between indigo and PB in oil paintings can be difficult since both pigments exhibit very similar properties [12].

Several decades ago an interesting approach for identification of three major nineteenth-century blue colorants including Prussian blue and indigo was proposed [13]. After digestion with sulfuric acid and extraction Prussian blue was confirmed through wet chemical analysis for ferrocyanide ions and indigo was examined with UV/Vis and IR spectroscopy. The recent protocol for surface enhanced Raman spectrometry (SERS) identification of both colorants was based on similar sample treatment by sulfuric acid [14]. LDI-MS, IR and Raman spectroscopy were compared for identification of PB, indigo and copper phthalocyanine in fresh and artificially aged samples. LDI-MS detected the lowest content of pigments with one exception – PB in the aged sample [15]. Other MS methods were reported but not for simultaneous analysis of both pigments. Therefore, the issue of a simple and fast method for identification of these both pigments still remains relevant.

2.2 Differentiation of plant gum binders by SFC/MS

2.2.1 Applications of plant gums

Plant-derived gums have been used as adhesives, binders, thickening, gelling, emulsifying and stabilizing agents for many ages. Traces of a plant gum were found in Egyptian mummies dated as early as 5th millennium BC [16]. The most well-known representative is gum Arabic derived from *Acacia* trees due to its practical and industrial importance. Other widely used binders include fruit tree gums: cherry (*Prunus avium*), peach (*Prunus persica*), plum (*Prunus* sp.), apricot (*Prunus* sp.) and some other exudates: tragacanth (*Astragalus* sp.), carob (*Ceratonia siliqua*), guar (*Cyanoposis tetragonolobus*), ghatti (*Anogeissus* sp.) and karaya (*Sterculia* sp.) [17].

Since gums are water-soluble materials or at least swell in water, they found extended application in water-based painting media, such as aquarelle, gouache, gum tempera as well as metallo-gallic inks [17,18]. Plant gums have also been used in dry painting media (pastels, pencils, charcoal). Gum

Arabic and tragacanth were applied in antiquity as binding media for pigments in Egyptian ointments used for mummification, in mural paintings in Christian catacombs, in paintings on silk and in manuscript illumination in the Middle Ages [17]. Identification of a particular binder is useful for restoration purposes as well as for evaluation of object authenticity and its possible provenance.

2.2.2 Analysis of plant gum-based binders

Traditional protocol for analysis of plant gum-based binders includes hydrolysis of polysaccharide material to simple monosaccharides and their separation following appropriate detection, although IR and Raman spectroscopy of intact binders are also utilized [17,19]. The number of sugars encountered in plant gums is relatively limited: L-arabinose (Ara), D-xylose (Xyl), L-fucose (Fuc), L-rhamnose (Rha), D-glucose (Glc), D-Galactose (Gal), D-Mannose (Man) and two sugar acids, D-glucuronic acid (GlcA) and D-galacturonic acid (GalA). Classification is based mainly on qualitative information (decisional scheme) [20-22]. Direct quantitation is often complicated. Additionally, the composition of an art or historical sample might be affected by different factors: presence of other saccharide materials, biological attack and aging [22,23]. For example, Xyl and Man might derive from softwood. Egg binder interferes with Man identification. Rha and uronic acids are subjected to degradation in the presence of certain pigments. Therefore, alternative methods utilizing relative ratio of monosaccharides [24], principal component analysis (PCA) [25,26] and cluster analysis [26] were applied for discrimination between plant gums.

2.2.3 Separation of monosaccharides

The earliest techniques available for separation of monosaccharides were paper chromatography and TLC. Later they have been replaced by more sophisticated methods, such as LC and GC. Normal-phase and HILIC modes of LC are preferred for native sugars. For instance, a Supelcosil NH_2 column with acetonitrile/water (80:20) as a mobile phase was used for characterization of neutral monosaccharides composition of *Acacia* exudate gums [27]. Alternatively, anion exchange chromatography was utilized with minimal sample treatment [17,25,28,29]. GC was used after derivatization of polar non-volatile compounds [17,24,26,30]. CE requires derivatization or highly alkaline medium to ionize neutral sugars as well as high concentration of background electrolytes [17,21].

SFC was developed mainly for non-polar analytes, although its range of application can be expanded by appropriate modifiers and additives. Few papers on SFC of carbohydrates with ELSD detection were published in mid-90s [31]. Among sugars of our interest, Rha, Xyl, Fru, Man and Glc were separated on a LiChrospher diol column with CO_2/methanol (84.5:15.5, v/v) mobile phase. Rha, Fru and Glc were resolved on a RSil NO_2 column with CO_2/methanol (87:13, v/v). A LiChrosorb CN column allowed identification of Fru and Glc with CO_2/methanol gradient. Zorbax Sil (silica) separated Rib, Rha, Fru, Man and Glc with CO_2/modifier (87:13, v/v), where modifier was a mixture of methanol/water/pyridine/triethylamine (91.95:4:4:0.05, v/v). Zorbax Sil and trimethylsilyl (TMS) phases were tested with methanol/water modifier (0, 4 and 8% of water) [32]. Separation of monosaccharides improved comparing to pure methanol, but pairs Ara-Xyl and Gal-Glc remained only partially resolved on both columns. Other saccharides useful for plant gum identification (Fuc, GlcA and GalA) were not included in any of the mentioned works on SFC.

Therefore, we aimed to push further the limits of SFC and investigate its applicability for analysis of all monosaccharides encountered in plant gum binders.

2.3 SFC/MS method for analysis of polar designer drugs

2.3.1 New designer drugs

Synthetic cannabinoids, phenylethylamines and cathinones are the most common constituents of “legal highs” [33]. The latter two classes of drugs together accounted for 42% of reports in period from 2008 to 2013. There has been a 60-fold increase in the number of seizures of synthetic cathinones in Europe during this time [34]. Since the trend of cathinones expansion will likely persist in the near future, development of new analytical methods for their identification is urgently required by analytical chemists, forensic experts, toxicologists and physicians.

2.3.2 Analysis of new designer drugs

Until recently, a two-step analytical strategy has been used by the forensic toxicology laboratories [35,36]. The first step included preliminary high throughput screening method (color tests, immunoassay) aimed at identification of several classes of compounds. The second, confirmation step was based on a highly selective technique able to provide accurate

recognition and quantitation of the target compounds (GC/MS). More recently, high throughput techniques based on LC coupled to triple quadrupole or to ion trap mass spectrometers prompted their incorporation into both steps of drug analysis strategy [35-38]. GC and CE methods were also reported, although for a limited number of applications. High resolution LC/MS was successfully tested for untargeted screening of drugs without the availability of primary reference standards.

Resolution of isomers remains an important issue in identification of NDDs. Among 125 cathinones ever reported by this time [39], 89 substances have at least one isomeric pair with identical monoisotopic mass. The analysis of positional isomers of fluoromethcathinone revealed that their retention times in GC were very similar, while the fragmentation patterns were almost identical [40]. LC/MS screening of methylated and fluorinated phenylethylamine and cathinone positional isomers with similar retention times and identical $[M+H]^+$ ions was also reported as challenging [41].

So-called ultra-high performance supercritical fluid chromatography (UHPSFC) employing columns with sub-2 μm particles, combines benefits of both SFC and UHPLC and can be advantageous over other techniques especially in those areas where high-throughput separation is required, such as screening analysis of NDDs. Attempts to use SFC for separation of illegal drugs, particularly amphetamines, were described as early as in 1990 [42]. The drawback of the method was necessary derivatization of amino group. In the later paper on stimulants addition of 0.5% isopropylamine to methanol modifier was crucial for efficient separation [43]. Basic additive, 0.5% cyclohexylamine in isopropanol, was beneficial for chiral separation of amphetamine and methamphetamine enantiomers [44]. Amphetamine, MDA, MDEA and MDMA were included in the test set of hydrophilic drugs investigated on UHPSFC system, but no detailed chromatographic data is available on these compounds [45]. Interestingly, recently published reviews highlight the importance of chromatography and mass spectrometry in analysis of NDDs but do not mention any applications of SFC [35-37]. By this time no dedicated papers on SFC of cathinones were published.

Therefore, purpose of this work was to test applicability of UHPSFC for analysis of cathinones and phenylethylamines with emphasis on resolution of isomeric compounds that cannot be unambiguously distinguished by MS even employing highly specific SRM mode.

3 AIMS OF THE THESIS

The aim of this thesis was to develop new mass spectrometric methods for application in the field of forensic science. Particular tasks included:

- sensitive detection of historical pigments indigo and Prussian blue in paintings;
- analysis of natural polysaccharide-based binders in painting medium;
- identification of new designer drugs (cathinones and phenylethylamines) with emphasis on distinguishing isomeric substances.

The first and second tasks concern issues of authenticity, origin and dating of artworks and historical artifacts while the third one deals with modern drugs of abuse. The main focus of this study was placed on sensitive detection, although simple and elegant sample preparation was also paid enough attention. Last but not the least, SFC separation conditions were investigated in details.

4 EXPERIMENTAL PART

4.1 Identification of Prussian blue and indigo by FIA/ESI-MS

Chemicals and samples

Prussian blue, PB, was synthesized from iron (III) chloride and potassium hexacyanoferrate (II). Other chemicals were of analytical grade. Solvents were HPLC grade or better. Samples of two oil paintings (beginning of the 20th century, 'Blue 1' and 'Blue 2') and a microsample from the painting 'Crucifixion', the St. Šebestián church on St. Hill, Mikulov, the Czech Republic, were provided by IMAGO v.o.s. (Mikulov, Czech Republic).

Pigment standards were sonicated in an alkaline solution. PB decomposed, suspended indigotin was further reduced by addition of sodium dithionite. Solutions were filtered and diluted in HPLC vials. A microsample of the painting 'Crucifixion' was treated directly in a vial due to its very small amount (unweightable, < 50 μg).

FIA/MS conditions

An Acquity UPLC system (Waters, Manchester, UK) was used for flow-injection experiments. Water/methanol (50:50, v/v) at flow rate 0.075 ml/min was used as a carrier liquid. The injection volume was 5 μl. A PEEK capillary (0.25 mm I.D., 20 cm length) replaced a chromatographic column for FIA. A Q-TOF Premier mass spectrometer equipped with a Z-spray ESI source (Waters, Manchester, UK) monitored negative ions in full scan mode. Data acquisition and processing were performed by MassLynx v. 4.0 software (Waters, Manchester, UK). Linear regression analysis was carried out using QC-Expert v. 3.2 software (TriloByte LTD, Pardubice, Czech Republic). LODs were determined according to ICH Q2(R1) [46].

4.2 Differentiation of plant gum binders by SFC/MS

Chemicals and samples

Carbon dioxide was 4.8 grade (99.998%). Water and methanol were LC/MS grade. Monosaccharides (**Fig. 4.1**) and other chemicals were of p.a. quality or better. Arabic, cherry and tragacanth gums were obtained from Kremer Pigmente (Aichstetten, Germany). Burnt Sienna, Sap Green and Ultramarine Blue aquarelles (Pébéo, Gemenos, Cedex, France) were bought

at a local art supplies shop in Olomouc. Samples of ceramic fragments with colored pattern were kindly provided by Martin Monik, Department of Geology, Faculty of Science, Palacký University.

Gums, aquarelle samples and paint from ceramic fragments were sonicated in aqueous TFA and hydrolyzed for approx. 4 minutes in vials with punctured septa caps placed into a beaker with water in a domestic microwave oven (700 W). Hydrolysis was stopped after the beginning of boiling. Hydrolysates were filtered and diluted 10 times with deionized water.

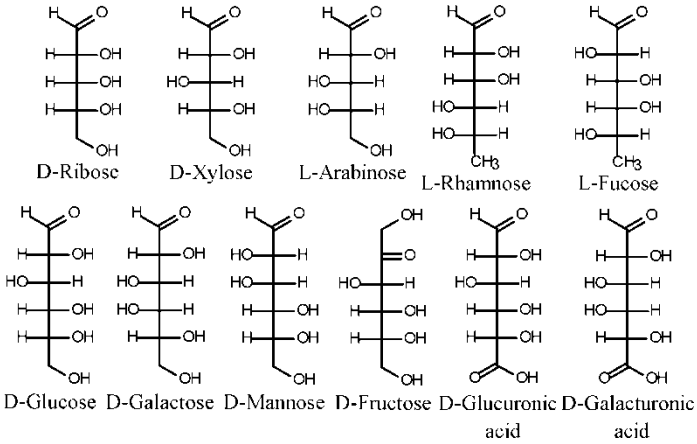


Figure 4.1 Structures of the investigated saccharides

Instruments

An Acquity UPC^2 system coupled to a Xevo TQD triple quadrupole mass spectrometer with a Z-spray electrospray source (all from Waters, Manchester, UK) was used. Make-up liquid was delivered by a 515 HPLC Pump operated via a Pump Control Module II (both from Waters, Manchester, UK). Control of the instruments and data acquisition was performed using Waters MassLynx 4.1 (Waters, Manchester, UK).

Continuous polarity switching was used during the method development and sample analysis. Water/methanol (50:50, v/v) containing 10^{-6} M sodium acetate at flow rate 0.400 ml/min was used as a make-up liquid. SRM transitions are listed in **Tab. 4.1**. Losses of water and carbon dioxide were excluded due to the low selectivity.

Table 4.1 List of SRM transitions.

#	Substance	Formula	MI mass	Precursor	Cone, V	SRM product[b)]	CE, V
1	galactose	$C_6H_{12}O_6$	180.06339	163.00	26	91.00 (Man)	10
2	fucose	$C_6H_{12}O_5$	164.06847	181.97	16	74.96	12
3	mannose	$C_6H_{12}O_6$	180.06339	198.00	20	127.00 (Fru, Gal)	16
4	pentoses	$C_5H_{10}O_5$	150.05283	323.00	15	173.00	10
5	deoxyhexoses	$C_6H_{12}O_5$	164.06847	350.91	20	186.99	10
6	hexoses	$C_6H_{12}O_6$	180.06339	382.97	22	202.99	10
7	uronic acids[a)]	$C_6H_{10}O_7$	194.04265	192.88	26	112.87 88.90 59.00	12 10 16

a) negative mode
b) interfering compounds

Chromatographic conditions and design of the experiment

Four Waters Acquity UPC^2 stationary phases were tested: **BEH** (silica) 1.7 μm, BEH **2-EP** (2-ethylpyridine) 1.7 μm, CSH **Fluoro-Phenyl** 1.7 μm and HSS **C18SB** 1.8 μm, all 100×3 mm i.d. Effect of water, ethanol, acetonitrile, formic acid, acetic acid, TFA, ammonium hydroxide and ammonium formate was investigated. Final conditions: ABPR pressure was 2000 psi (138 bar), column temperature was 35 ºC. Modifier consisted of methanol/water/formic acid (91:5:4 v/v/v). Elution program for BEH: initial – 5%; 9 min – 20%; 10 to 11 min – 5% modifier, flow rate – 2.5 ml/min. Elution program for C18SB: initial – 0%; 5 min – 30%; 6 to 7 min – 0% modifier, flow rate – 2.0 ml/min. Mixture (2 μl) containing all monosaccharides and uronic acids (2×10^{-5} M each) or a sample was injected. Principal component analysis (PCA) was performed by means of OriginPro 2015 software (OriginLab Corporation, Northampton, MA, USA).

4.3 SFC/MS method for analysis of polar designer drugs

Chemicals and instruments

Solvents were the same as in section **4.2**. Standards and other chemicals (**Fig. 4.2**) were of analytical grade purity. Working solutions were prepared in methanol.

Instruments are described in section **4.2**. Methanol was used as a make-up liquid and delivered at 0.400 ml/min flow rate. SRM transitions are listed in **Tab. 4.2**.

Chromatographic conditions and design of the experiment

Stationary phases are described in section **4.2**. Mobile phase flow rate and ABPR were set to 2.0 ml/min and 2000 psi (138 bar), respectively. Six modifiers (pure methanol, 2% water in methanol, 20 mM ammonium hydroxide, 20 mM ammonium formate, 20 mM ammonium acetate and 20 mM formic acid in methanol) were utilized. Combination of 2% water and 20 mM ammonium formate in methanol was examined on HSS C18SB and CSH Fluoro-Phenyl columns. Additive concentration was investigated on BEH and Fluoro-Phenyl columns with 5, 10 and 20 mM ammonium hydroxide and ammonium formate in methanol. 40, 50 and 60 °C column temperatures were tested. Gradient program was set as follows: initial – 6%; 5 min – 30%; 6 to 7 min – 6% of modifier. Mixture (2 µl) containing all 15 substances (500 ng/ml each) was injected. The peak width at half maximum ($W_{1/2}$) and asymmetry at 10% peak height were calculated using TargetLynx software (Waters, Manchester, UK). Peaks were considered Gaussian for asymmetry factor in the range 0.8 – 1.4.

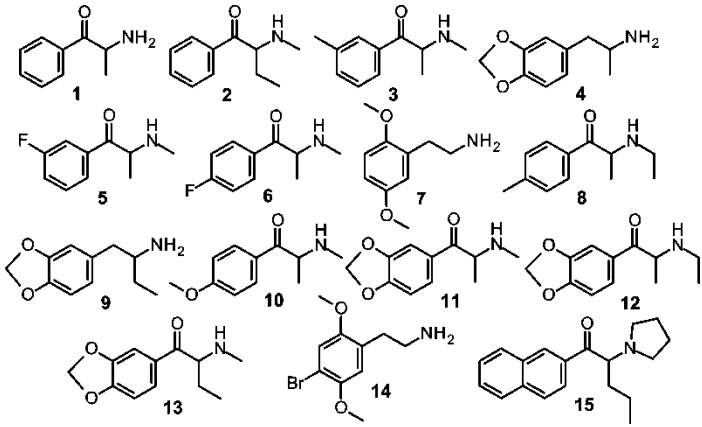


Figure 4.2 Investigated substances: (**1**) cathinone; (**2**) buphedrone; (**3**) 3-MMC; (**4**) MDA; (**5**) 3-FMC; (**6**) flephedrone; (**7**) 2C-H; (**8**) 4-MEC; (**9**) BDB; (**10**) methedrone; (**11**) methylone; (**12**) ethylone; (**13**) butylone; (**14**) 2C-B; (**15**) naphyrone.

Table 4.2 List of investigated substances and their SRM transitions.

#	Substance	Formula	MI mass	Precursor	Cone V	SRM product*	CE, V
1	cathinone	$C_9H_{11}NO$	149.08406	149.92	30	**116.97**	**18**
2	buphedrone	$C_{11}H_{15}NO$	177.11536	177.96	28	**130.96** (**3**)	**24**
3	3-MMC	$C_{11}H_{15}NO$	177.11536	177.96	30	**144.94** (**2**)	**20**
4	MDA	$C_{10}H_{13}NO_2$	179.09462	180.00	20	**104.96**	**22**
5	3-FMC	$C_{10}H_{12}NOF$	181.09028	181.93	32	**122.96** (**6**)	**20**
6	flephedrone	$C_{10}H_{12}NOF$	181.09028	181.93	32	**148.98** (**5**)	**20**
7	2C-H	$C_{10}H_{15}NO_2$	181.11027	181.95	14	**149.97** (**14**)	**18**
8	4-MEC	$C_{12}H_{17}NO$	191.13101	191.97	30	**144.20**	**28**
9	BDB	$C_{11}H_{15}NO_2$	193.11027	194.01	24	**134.93** (**10**)	**14**
10	methedrone	$C_{11}H_{15}NO_2$	193.11027	194.01	28	**161.00**	**20**
11	methylone	$C_{11}H_{13}NO_3$	207.08953	207.93	18	**159.97**	**16**
12	ethylone	$C_{12}H_{15}NO_3$	221.10518	222.01	32	**174.12** (**13**)	**18**
13	butylone	$C_{12}H_{15}NO_3$	221.10518	222.01	32	**174.12** (**12**)	**18**
14	2C-B	$C_{10}H_{14}NO_2Br$	259.02078 261.01873	259.86	20	**227.85**	**22**
15	naphyrone	$C_{19}H_{23}NO$	281.17796	282.08	40	**140.96**	**22**

* interfering compounds are listed in brackets.

5 RESULTS AND DISCUSSION

5.1 Identification of Prussian blue and indigo by FIA/ESI-MS

Part of materials presented in this section has been published in ref. [47].

5.1.1 Method development

PB (solubility product around 10^{-40}) and indigo are insoluble in water and common organic solvents [48,49]. Solvents like DMSO, DMF, THF and concentrated HCl are often used for indigo extraction from paintings and plant materials, but they are incompatible with ESI [2]. To overcome low solubility, PB was quantitatively decomposed in alkaline solution (pH≥11) to form iron (III) hydroxide and hexacyanoferrate (II) ions (**Fig. 5.1, a**) that can be detected by MS. Indigotin was reduced to leucoindigo (LI) by 3 mg/ml sodium dithionite at eventual pH=12.3 (**Fig. 5.1, b**).

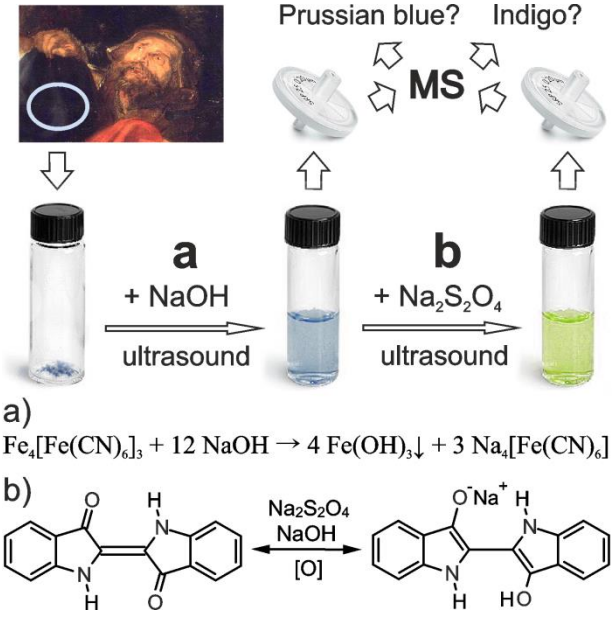


Figure 5.1 Protocol for analysis of paint microsamples: **a)** decomposition of PB; **b)** conversion of indigotin to soluble LI (adopted from [47]).

A spectrum of decomposed PB sample was in accordance with an ESI-MS spectrum of potassium hexacyanoferrate (II) (**Fig. 5.2, a**) and literature data [50]. MS/MS spectrum is shown in **Fig. 5.2, b**. Besides accurate masses, a typical isotopic profile of Fe proved the composition of ions. Ferrous complexes with CN^- ligand were essential for PB identification. Only iron detection would not be sufficient as other ferrous pigments can be present, e.g. in multilayer paintings. LI produced $[M-H]^-$ ion (**Fig 5.3, a**). Its fragmentation rendered product ions useful for identification (**Fig. 5.3, b**).

Simultaneous analysis of both pigments within one spectrum was evaluated. After treatment with sodium dithionite an unidentified peak around m/z 133.946 interfered with PB signal in all samples and blank. The problem was solved by the separate analysis of sample solution prior and after addition of sodium dithionite.

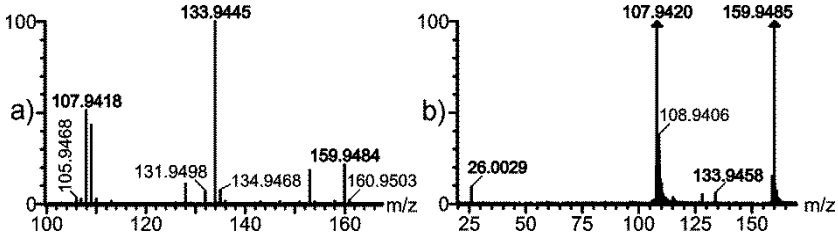


Figure 5.2 a) Mass spectrum of potassium hexacyanoferrate (II) 10 μg/ml in water; **b)** fragmentation spectrum of m/z 159.9485.

The automatic flow-injection analysis preserved samples from being re-oxidized by air oxygen and provided short runtimes. The largest peak area without peak tailing compromising detection was achieved at flow rate 0.075 ml/min with analysis time below two minutes (**Fig. 5.4**).

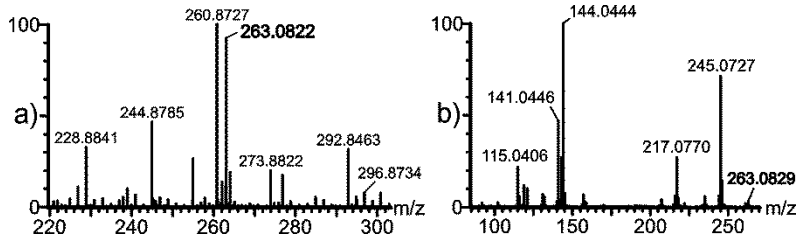


Figure 5.3 a) Mass spectrum of LI; **b)** fragmentation spectrum of LI.

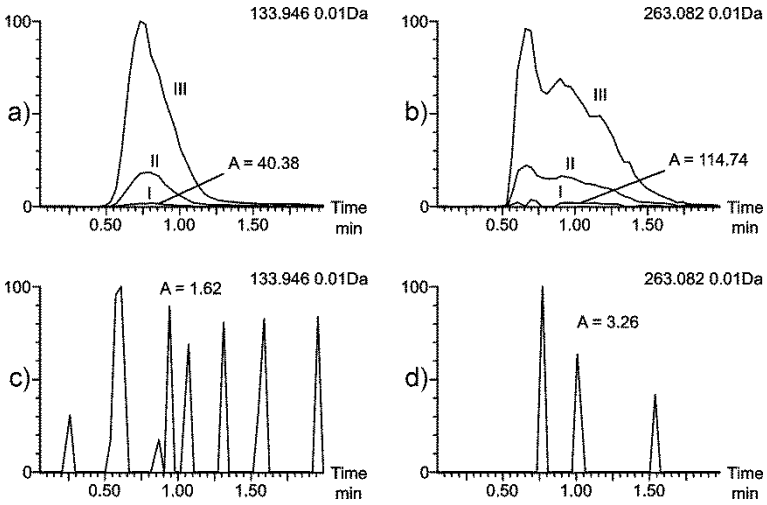


Figure 5.4 FIA mass-chronograms: **a)** PB, **b)** indigo. Curves I, II, III correspond to 100, 1000 and 5000 ng/ml concentrations, respectively; **c)** and **d)** are corresponding blanks; integrated peak areas are shown.

Calculated LODs for PB and indigo were 10 ng/ml (47 pg) and 12 ng/ml (59 pg), respectively. Calibration curves were linear up to 5000 ng/ml. For indigotin, the linearity range and LOD were comparable to an HPLC/MS method (30–4200 ng/ml, 50 pg on column) [51].

5.1.2 Analysis of samples from oil paintings

LOD values indicated the feasibility of the developed protocol for real samples. It was further tested using two blue samples of oil paintings from the 20th century. 'Blue 1' was identified as PB with no detectable traces of indigo, 'Blue 2' contained indigo but not PB, both in agreement with the known sample composition.

Blue paint was sampled to examine the painting 'Crucifixion' from the St. Šebestián Church on Saint Hill in Mikulov, the Czech Republic. The painting is a palimpsest with an older layer different in arrangement of figures, allegedly dated to the 16th century. Electron probe microanalysis excluded blue pigments widely used from Middle Ages up to the 19th century like ultramarine, azurite, blue verditer, vivianite, smalt etc. Neither electron probe microanalysis nor Raman spectroscopy has rendered a clue for PB or indigo identification. Utilizing the developed protocol, no traces of indigo

were found in the microsample of the painting 'Crucifixion', but the presence of PB was evident. The result of analysis supports the hypothesis that the object was re-painted after the 18th century or later.

5.1.3 Conclusion

The developed FIA/ESI-MS protocol proved to be successful in identification of pigments of high historical importance – PB and indigo. It offers indigotin LOD comparable to HPLC/MS. To our best knowledge, this is the only ESI-MS method for simultaneous identification of PB and indigo by the moment of the publication. It is rapid, simple, sufficiently sensitive and allows for mass spectrometric analysis without chromatographic separation. It represents a useful alternative to other methods applied in simultaneous analysis of both pigments. The microsample of the painting 'Crucifixion' contained PB which could exclude Middle Ages origin of the painting. Since the painting is a palimpsest with cracks, analysis of other samples from different locations is suggested to confirm PB in both layers.

5.2 Differentiation of plant gum binders by SFC/MS

5.2.1 Method development

The observed monosaccharide precursor ions were in agreement with the literature [52]. Generally, pentoses, hexoses and deoxyhexoses produced sodium adducts $[M+Na]^+$ and sodiated dimers $[2M+Na]^+$. $[M+Na]^+$ ions gave no useful fragmentation while $[2M+Na]^+$ ions lost one monosaccharide moiety and $[M+Na]^+$ products were detected in MS/MS experiments. Uronic acids produced deprotonated ions in negative mode. Additional specific transitions are listed in **Table 4.1**.

Appropriate modifier was crucial for successful separation of such polar analytes. Among the tested additives mixture of 5% water and 4% HCOOH provided the best peak shapes, separation between individual monosaccharides and complete elution of uronic acids except from the 2-EP column, where strong electrostatic interaction occurred.

At 35 ºC several sugars provided two peaks corresponding to α and β anomers. Whilst one of the anomers overlapped with other sugar, the second could be used for identification. At 60 ºC anomers merged into one peak and thus, identification of individual sugars became impossible.

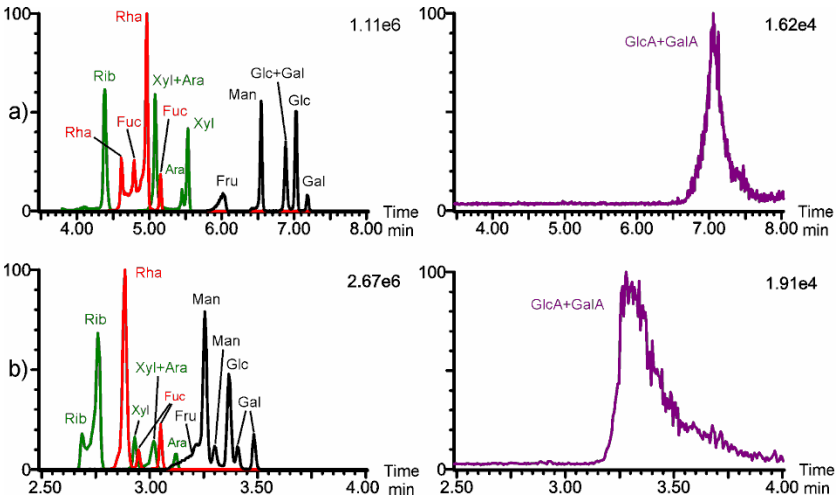


Figure 5.5 Optimized SFC separation of the standard mixture: **a)** BEH, gradient: 5 to 20% modifier in 9 min, 2.5 ml/min; **b)** C18SB, gradient: 0 to 30% modifier in 5 min, 2.0 ml/min.

Retention of monosaccharides on all phases strongly correlated with the number of hydroxyl groups. Thus, pentoses and deoxyhexoses were eluted first, then hexoses and, finally, uronic acids. This elution order is in agreement with the literature [31] and strongly resembles a typical behavior of sugars in HILIC [29]. Uronic acids, however, were not separated and they were eluted as a broad band due to high polarity and presence of deprotonated form. We observed three critical pairs common to all stationary phases: Fuc-Rha, Ara-Xyl and Gal-Glc. After tuning of chromatographic conditions, BEH and C18SB clearly outperformed other stationary phases and allowed identification of each monosaccharide (**Fig. 5.5**).

5.2.2 Analysis of plant gums and samples

A sample of paint from ceramic fragments dated to the 14th century and a commercial set of watercolors were analyzed. Paint from ceramic fragments contained only Glc and Fru in 94.7:5.3 ratio (C18SB, RSD<1%). Combination of these two monosaccharides may be indicative for flour, honey or fruit juice. Monosaccharides common to gum Arabic and fruit tree gums were found in all aquarelle hydrolysates. It was impossible to attribute clearly the quantitative data to a specific plant gum.

We tried to classify plant gums and watercolor samples on the basis of their monosaccharide ratios: Rha to (Ara+Xyl), Fuc to (Ara+Xyl) and Gal to (Ara+Xyl) [24]. Unusually high values for Glc found in samples may indicate presence of other saccharide materials. Therefore, Glc was omitted from comparison. Results from C18SB (**Fig. 5.6**) showed distribution similar to the literature data [24]. Numerical values were in agreement with statistics from the same literature source. Aquarelles fell within the group of cherry gum.

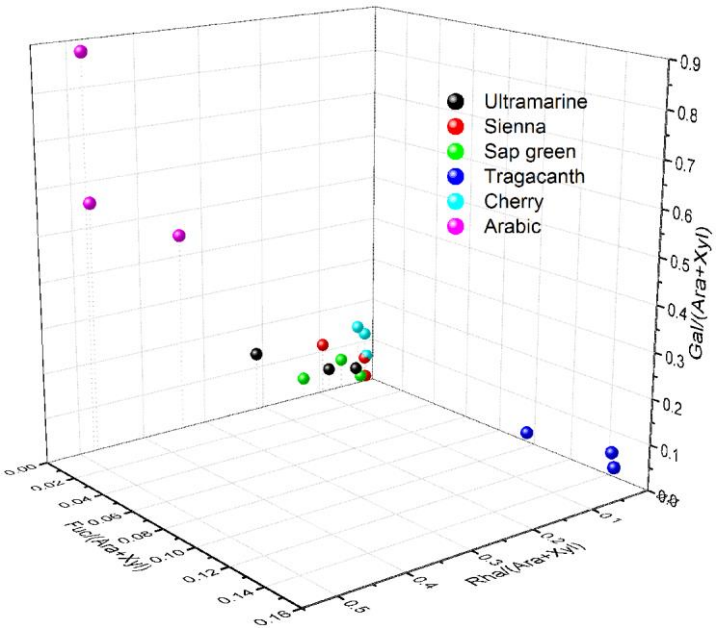


Figure 5.6 Classification of plant gums and aquarelle samples based on SFC/MS peak area ratios on the C18SB column

PCA analysis was based on the relative peak areas of monosaccharides as variables. BEH and C18SB columns enabled clear grouping of plant gum samples. Aquarelles did not overlap with any of the plant gums. Their position was correlated with Glc on the loading plot. After exclusion of Glc, which may be present due to contamination or another saccharide material, group of aquarelles moved towards the cherry gum (**Fig. 5.7)**. A binder from an alternative source could be used for manufacturing of

aquarelles, most likely another *Prunus* sp. More gum samples from different sources are needed for objective comparison.

PCA provided more precise results comparing to the classification on the basis of peak area ratios. In the latter case binder in the aquarelle samples might be mistakenly attributed to cherry gum. The reported results are comparable with PCA based on GC/MS data [30].

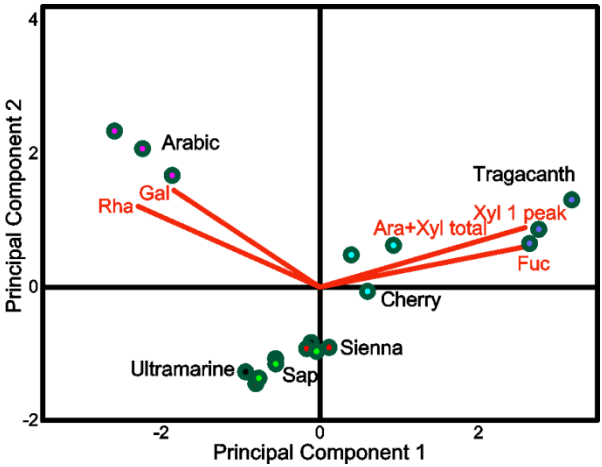


Figure 5.7 PCA biplot of plant gums and aquarelles based on selected variables, C18SB column.

5.2.1 Conclusion

Water with formic acid as components of modifier provided increase in the elution strength of SFC mobile phase and allowed separation of nine monosaccharides and elution of very polar sugar acids. The C18SB column showed the best results in terms of monosaccharide separation, analysis time and sensitivity. The developed SFC/MS method was applied for examination of plant gums used as binders in painting media. Analyzed samples were successfully classified by PCA on the basis of selected monosaccharide relative peak areas. Gum Arabic, cherry gum and tragacanth were clearly distinguished from each other. Aquarelles did not fell in any gum group and occupied a separate zone. A binder from another source could be used for manufacturing of aquarelles (*Prunus* sp.). Sample of paint from historical ceramic fragments contained only Glc and Fru. This combination may indicate use of flour, honey or fruit juice.

5.3 SFC/MS method for analysis of polar designer drugs

Part of materials presented in this section has been accepted for publication: V. Pauk, V. Žihlová, L. Borovcová, V. Havlíček, K. Schug, K. Lemr, *J. Chromatogr. A* (2015).

5.3.1 Method development

A single most intensive specific SRM transition was monitored (**Tab. 4.2**). Four pairs of isomers could not be unambiguously distinguished by MS: buphedrone (**2**) and 3-MMC (**3**); 3-FMC (**5**) and flephedrone (**6**); BDB (**9**) and methedrone (**10**); ethylone (**12**) and butylone (**13**) due to the interfering SRM transitions.

Among all tested additives ammonium hydroxide, formate and acetate delivered the best results. All compounds were eluted in 3.5 minutes or less and most of the peaks had Gaussian shape. The best results were reached using 20 mM concentration of the additives. Addition of 2% water to ammonium formate modifier allowed to resolve BDB (**9**) and methedrone (**10**) on the Fluoro-Phenyl column and improved separation of ethylone (**12**) and butylone (**13**) on C18SB. These additives ensured high detection sensitivity, improved peak shape, provided faster elution of analytes but did not change significantly the selectivity of separation. Retention factors were highly correlated on all used columns ($r^2 \geq 0.984$).

Table 5.1 Evaluation of stationary phases.

St. phase, additive	T, °C	Isomers		All compounds					
		$R>1$	$1>R>0.3$	$R>1$	$1>R>0.3$	$R<0.3$	Gaus.	Time min	$W_{1/2}$, s
BEH, NH_4OH	40	4	0	11	2	2	15	2.87	2.51
BEH, $HCOONH_4$,	40	4	0	11	2	2	10	3.25	2.61
Fl-Ph, NH_4OH	60	4	0	10	4	1	11	2.36	1.99
Fl-Ph, $HCOONH_4$, 2% H_2O	60	4	0	10	4	1	10	2.62	2.34
C18, $HCOONH_4$, 2% H_2O	60	3	1	12	2	1	13	3.67	2.30
2-EP, NH_4OH	40	2	1	6	5	4	10	2.08	2.29

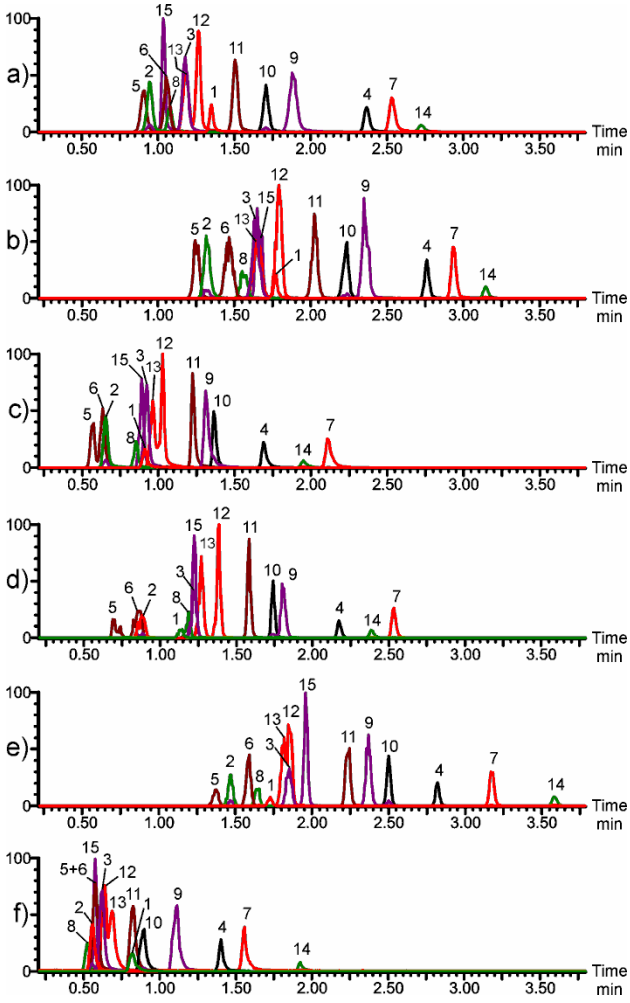


Figure 5.8 The most suitable separation conditions for an each stationary phase: **a)** BEH 40 °C, 20 mM NH_4OH; **b)** BEH 40 °C, 20 mM $HCOONH_4$; **c)** Flouro-Phenyl 60 °C, 20 mM NH_4OH; **d)** Flouro-Phenyl 60 °C, 20 mM $HCOONH_4$, 2% H_2O; **e)** C18SB 60 °C, 20 mM $HCOONH_4$, 2% H_2O; **f)** 2-EP 40 °C, 20 mM NH_4OH.

The primary criterion, resolution of isomers, was fulfilled only by two phases: BEH and Fluoro-Phenyl (**Tab. 5.1, Fig. 5.8**). Three pairs showed resolution ≥2.0 on BEH at 40 ºC. Ethylone (**12**) and butylone (**13**) were more

resolved on Fluoro-Phenyl. The highest resolution for this pair, as well as for BDB (**9**) and methedrone (**10**), on Fluoro-Phenyl was achieved by addition of 2% water to methanolic ammonium formate. C18SB failed to resolve ethylone (**12**) and butylone (**13**) regardless all investigated experimental conditions. 2-EP did not separate 3-FMC (**5**) and flephedrone (**6**) and had insufficient resolution for ethylone (**12**) and butylone (**13**).

Under the most suitable conditions 15 drugs were separated on BEH in less than 3 min. Increase of the flow rate and gradient slope reduced analysis time to 1.6 min and maintained acceptable separation of isomers (**Fig. 5.9**).

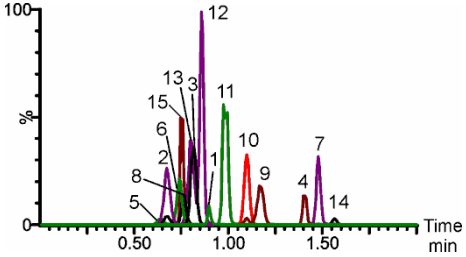


Figure 5.9 Ultra-fast analysis on the BEH column, 40 °C, 20 mM NH_4OH, 6-24% in 1.5 min, 2.9 ml/min, max. pressure 400 bar, ($R_{2,3}$=1.87; $R_{5,6}$=1.37; $R_{9,10}$=0.99; $R_{12,13}$=0.84).

5.3.2 Retention correlation

All investigated stationary phases showed very similar selectivity. The retention factors strongly correlated on all phases ($r^2 \geq 0.845$). Flephedrone and 2C-B possessing fluorine or bromine atoms, respectively, were less retained on Fluoro-Phenyl. Electronegative atoms attract electrons from aromatic ring which might decrease π-π interaction between the stationary phase and analytes. A decrease in the polarity of stationary phases (2-EP>BEH>Fluoro-Phenyl>C18SB) resulted in lower selectivity for BDB (**9**) and methedrone (**10**), and a reversed elution order was observed on the C18SB phase.

We observed several structure-related correlations in behaviour of investigated compounds (**Fig. 5.8**). Longer alkyl chain at or in close proximity to amino group caused shorter retention of cathinones ((**12**) < (**11**), (**13**) < (**11**), (**8**) < (**3**), (**2**) < (**1**)), as well as of phenylethylamines ((**9**) < (**4**) < (**7**)). 2C-H (**7**) and 2C-B (**14**) without an alkyl group in the α-position were

the most retained substances. Therefore, accessibility of the nitrogen atom in amino group is directly related to retention of a particular substance. The presence of methylenedioxy group increased polarity and retention of cathinones ((**2**) < (**13**)). A similar effect was observed with the substitution of 4-methyl by 3,4-methylenedioxy group ((**8**) < (**12**)) in contrast to substitution of 4-methoxy by 3,4-methylenedioxy group, which decreased the actual retention ((**11**) < (**10**)). Fluorine-substituted cathinones eluted faster than methyl ((**5**) < (**3**)), methoxy ((**6**) < (**10**)) or methylenedioxy substituted ones ((**6**) < (**11**)). In general, for very similar structures, a substance with higher log *P* and lower pK_a (less polar) should elute earlier. However, greater retention was observed for a 4-methoxy (**10**) contrary to a 3,4-methylenedioxy derivative (**11**) despite decrease of log *P* and increase of pK_a values. The elution order of (**7**) and bromine-substituted (**14**) also differed from the predicted behaviour, except in the case of the Fluoro-Phenyl phase. Isomeric pairs with different structures ((**2**) and (**3**), (**9**) and (**10**)) can be resolved on all stationary phases while separation of positional isomers possessing very similar characteristics ((**5**) and (**6**), (**12**) and (**13**)) was difficult, especially on C18SB and 2-EP phases.

5.3.3 Conclusion

This is the first report on the application of UHPSFC for separation of polar synthetic cathinones and strongly basic phenylethylamines. The four stationary phases, BEH, BEH 2-EP, CSH Fluoro-Phenyl and C18SB were tested under various conditions. Highly correlated retention factors were obtained using different additives as well as stationary phases. Substances with higher log *P* and lower pK_a eluted faster. Detailed evaluation revealed some structure features influencing retention. The accessibility of a nitrogen atom in an amino group had the greatest impact on retention of a particular substance. The best separation results were achieved using BEH phase at 40 °C and 20 mM ammonium hydroxide or ammonium formate as modifier. Alternatively, CSH Fluoro-Phenyl can be used at 60 °C with 20 mM ammonium hydroxide or ammonium formate and 2% water in methanol as modifier. Under these conditions, the four isomeric pairs were sufficiently separated and occasional co-eluting species were easily distinguished by MS. The analysis took less than 3.3 minutes and could even be reduced to 1.6 minutes on the BEH column for ultra-fast screening of NDDs.

6 CONCLUSION

Three mass spectrometric methods were developed for forensic applications. The first one, FIA/ESI-MS protocol, was useful in detection of important historical pigments – PB and indigo. This is the only ESI-MS method for simultaneous identification of PB and indigo by the moment of publication. This method is rapid, simple, sensitive and does not require chromatographic separation. It offers indigotin LOD comparable to HPLC/MS. It represents a useful alternative to other methods applied in simultaneous analysis of both pigments. The developed method showed presence of PB in the microsample of the oil painting 'Crucifixion'.

The second, SFC/MS method, was applied for analysis of saccharide binders in painting medium. Increased elution strength of SFC mobile phase allowed separation of nine monosaccharides and elution of very polar sugar acids. Sample of paint from the 14th century ceramic fragments contained Glc and Fru which may indicate usage of honey, flour or fruit juice as a binder. Profile of commercial aquarelles was compared with plant gums. Gum Arabic, cherry gum and tragacanth were clearly distinguished from each other by PCA. Aquarelles did not fell in any gum group and occupied a separate zone. An alternative plant source of the binder used in watercolors is suggested, most likely a fruit tree belonging to *Prunus* sp.

The third method was focused on analysis of NDDs. This is the first report on the application of UHPSFC for separation of polar synthetic cathinones. SFC conditions were extensively investigated and general trends in chromatographic behavior of polar drugs were established. Retention factors were highly correlated on all stationary phases using ammonium based additives. In general, substances with higher log *P* and lower p*K*a eluted faster. The accessibility of a nitrogen atom in an amino group had the greatest impact on retention time of a particular substance. Efficient separation of four isomeric pairs and most of remaining analytes (fifteen compounds in total) was achieved in less than 3 minutes on BEH phase. ESI ionization with a triple quadrupole analyzer in SRM mode provided an additional dimension for differentiation and sensitive detection of all investigated substances. The analysis time was further reduced to 1.6 minutes on the BEH column for ultra-fast screening of NDDs.

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Curriculum vitae

Name: Volodymyr Pauk
Year and place of birth: 1988, Uzhhorod, Ukraine
E-mail: volodymyr.pauk@gmail.com

Education:

2005–2009 Bc. study, Analytical Chemistry, Chemical Faculty, Uzhhorod National University. Thesis: *Ion selective electrode for determination of perchlorate*.

2009–2010 M.Sc. study, Analytical Chemistry, Chemical Faculty, Uzhhorod National University. Thesis: *Potentiometric sensor for determination of Chlorate (VII) ions*.

2010–now Ph.D. study, Analytical Chemistry, Faculty of Science, Palacký University in Olomouc. Topic: Forensic application of mass spectrometry.

Internships:

2009–2010 Research/Study stay, Institute of Chemistry, Faculty of Science, P. J. Šafárik University in Košice, Slovak Republic, 10 months (supervisor: prof. Yaroslav Bazeľ). Topic: Development of the new methods for the chlorates determination

2013 Research stay, Department of Chemistry, University of Washington, Seattle, WA, USA, 3 months (supervisors: prof. František Tureček, Michael Volný). Topic: Interfacing Droplets with Mass Spectrometry for Single Cell Analysis.

2014 Work stay, TSA-QC department, TEVA Czech Industries, Opava-Komárov, 3 months (supervisor: Lukáš Dvořák). Subject: Validation of analytical methods.

Projects:

2009 International Visegrad Fund, VSP 50910788 *Development of the new methods for the chlorates determination*.

2012 University Development Fund (FRVŠ), 2004/2012/G6 *Creation of new laboratory exercises utilizing non-aqueous environment for innovation of Practicals in Analaytical Chemistry*, team member.

2013 University Development Fund (FRVŠ), 1188/2013/G6 *Creation of new laboratory exercises of food analysis for innovation of Advanced Analytical Chemistry*, team member.

2014 Ministry of education, youth and sports (MŠMT), Kontakt LH14064 *Analytical tools for fast identification of new designer drugs*, team member.

Pedagogical activities:

2009–2010 Lectures and seminars in chemistry for students of middle school, total 8 weeks.

2011 Teaching of Practicals in Analytical Chemistry (ACH/ACC), 1 semester.

2012 Teaching of Applied Analytical Chemistry (ACH/ACHSB), 1 semester.

2013 Teaching of English for Chemists 2 (ACH/CHA2), 6 weeks.

Publications:

V. Pauk, V. Havlíček, B. Papoušková, P. Sulovský, K Lemr, Simultaneous identification of historical pigments Prussian blue and indigo in paintings by electrospray mass spectrometry, *J. Mass. Spectrom.* 48 (2013) 927–930. doi: 10.1002/jms.3228.

V. Pauk, P. Barták, K. Lemr, Characterization of natural organic colorants in historical and art objects by high-performance liquid chromatography, *J. Sep. Sci.* 37 (2014) 3393–3410. doi: 10.1002/jssc.201400650.

V. Pauk, V. Žihlová, L. Borovcová, V. Havlíček, K. Schug, K. Lemr, Fast Separation of Selected Cathinones and Phenylethylamines by Supercritical Fluid Chromatography, *J. Chromatogr. A* (2015), accepted for publication.

International conferences:

Analitika RB – 2010, Minsk, Belarus, 14-15.5.2010, poster: V. Pauk., Y. Bazel – Новый перхлорат-селективный электрод с пластифицированной мембраной (A new perchlorate-selective electrode with plastificated membrane), Abstract book, 47.

53rd Hungarian Spectrochemical conference, Hajdúszoboszló, Hungary, 30.6–2.7.2010, poster: V. Pauk, Y. Bazel, J. Balogh – Development of a new perchlorate-selective electrode.

IMMS 2012, Olomouc, Czech Republic, 29.4–3.5.2012, poster: V. Pauk, B. Papoušková, P. Sulovský, K. Lemr – Identification of historical pigments Prussian Blue and Indigo by FIA/ESI-MS, *Chemica* 49S (2012) 119. Best poster prize.

4th EuCheMS Chemistry Congress, Prague, Czech Republic, 26–30.8.2012, poster: V. Pauk, B. Papoušková, P. Sulovský, K. Lemr – Mass spectrometric approach to evaluation of historical paintings by identification of Prussian blue and indigo, *Chem. Listy* 106 (2012) s1143.

Other activities:

Member of the organizing committee of Advances in Chromatography and Electrophoresis & Chiranal conference 2012 – 2014.

Contract research for companies Waters, Contipro, Novato, Atotech, Synthesia and TEVA Czech Industries.

Consultation of master thesis: Veronika Žihlová, Lucie Pušová (successfully finished).

Reviewer of the bachelor and master theses.