Evaluation of HILIC in a metabolomic set-up: comparison with a reversed-phase type of column

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Introduction

Nowadays, metabolomic research is a booming section in the functional genomics world. More and more, LC-MS is considered as the indispensable tool for metabolomics, due to a combination of sensitivity, discrimination and identification power, and applicability to a broad range of metabolites. The optimisation of chromatography is a first vital step in developing an LC-MS tool. In plant extracts, chemical compounds have predominantly polar characteristics that complicate the separation on conventional reversed-phase columns. To that end, we made a comparison between an Atlantis dC18 column (Waters), specially designed to retain polar compounds, and an alternative hydrophilic interaction type of chromatography, namely the TSKgel Amide-80 column (Tosoh Bioscience).

Aim

Our aim was to evaluate the potential surplus value of hydrophilic interaction chromatography in the field of metabolomics. In function of this evaluation, a comparison was intended with a reversed-phase column, normally used in our research field.

Materials

HPLC Conditions:

Alliance 2695 (Waters®)
Columns:
- Atlantis™ dC18, 3µm, 150 x 2.1mm (Waters®)
- TSKgel Amide-80, 5µm, 250 x 2.0mm (Tosoh Bioscience)

Mobile Phase:
- Eluent A: 100% water + 0.1% formic acid
- Eluent B: 90/10 acetonitrile/water + 0.1% formic acid

Flow Rate: 200µl/min
Injection Volume: 50µl

MS Conditions:

Mass Spectrometer: Q-TOF micro (Waters®)
Ion Source: Lockspray™ in both positive and negative ion mode
Software: Masslynx 4.0, Quanlynx
m/z-range: 50-800
Capillary Voltage: 3000V
Cone Voltage: 30V

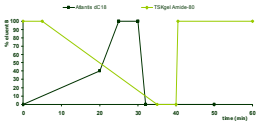
Metabolites:

Amino acids, carbohydrates, nucleotides, plant hormones, steroids, fatty acids, polyamines, carotenoids and others were used from stock solutions at 1mg/ml

Extraction procedure:

Arabidopsis thaliana leaves were homogenized with an Ultra-Turrax mixer (Ika) in 2:6:2 H_2O:MeOH:$CHCl_3$. Samples were centrifuged for 10 minutes and the supernatant (after spiking) was evaporated under nitrogen. Before injection, samples were dissolved in 75/25 H_2O/AcN + 0.1% FA.

Gradient Profile: (**optimised!**)



Methods

❖We developed a pure metabolite mix of 25 compounds representing the complex pattern of chemicals in an *Arabidopsis thaliana* plant, with log D values from -7.41 till 7.08. This mixture enables us to compare both columns from a metabolomics point of view.

❖The pure mix of metabolites (*pure*) was analysed next to an *A. thaliana* extract spiked with the metabolite mix (same concentration, *extract*). This was to investigate the impact of complex matrices on retention time and chromatographic performance. In metabolomics, relative variation on retention time is an important issue in data processing. Comparative sample analysis is only feasible with strongly reproducible LC-MS chromatograms.

Results

		Atlantis dC18							TSK Gel Amide 80						
		tR (mean)			RSD (%)		k'		tR (mean)			RSD (%)		k'	
metabolites	log D	pure	extract	tRe - tRp	pure	extract	pure	extract	pure	extract	tRe - tRp	pure	extract	pure	extract
lysine	-5.34	2.19	2.83	0.64	1.82	1.40	1.57	2.32	25.81	24.60	-1.21	0.18	1.10	13.42	12.74
leucine	-1.87	2.78	2.78	0.00	0.35	0.27	2.26	2.26	17.55	16.76	-0.79	0.36	0.73	8.80	8.36
serine	-3.58	2.27	2.54	0.27	1.71	1.17	1.66	1.97	21.90	21.68	-0.22	0.06	0.17	11.23	11.11
phenylalanine	-1.80	10.28	10.27	-0.01	0.32	0.24	11.05	11.04	17.35	16.63	-0.72	0.44	1.17	8.69	8.29
glutamylcysteine	-3.72	2.39	2.49	0.09	0.41	2.72	1.80	1.91	24.81	24.68	-0.13	0.18	1.09	12.86	12.79
S-adenosylmethionine	X	2.39	2.48	0.09	0.04	2.32	1.80	1.91	27.40	24.67	-2.73	0.33	1.05	14.31	12.78
lactose	-5.18	2.25	2.51	0.26	0.03	1.07	1.64	1.94	22.34	22.30	-0.04	0.02	0.09	11.48	11.46
galactose	-3.13	2.23	2.39	0.16	0.04	3.37	1.62	1.80	20.19	24.72	4.54	0.08	0.99	10.28	12.81
maltotriose	-7.24	2.54	2.66	0.11	0.34	1.20	1.98	2.11	23.62	23.46	-0.16	0.15	0.04	12.20	12.11
adenine	-0.61	2.78	2.76	-0.02	0.35	1.08	2.26	2.24	18.82	16.49	-2.33	0.66	2.94	9.51	8.21
uracil	-0.84	4.49	4.46	-0.03	0.78	0.15	4.26	4.23	4.51	4.43	-0.08	2.95	0.57	1.52	1.47
cytidine	-4.19	2.77	2.74	-0.03	0.07	0.97	2.24	2.21	22.04	20.75	-1.29	0.24	0.49	11.31	10.59
IMP	-4.59	2.80	2.78	-0.02	1.36	0.35	2.28	2.25	X	X	X	X	X	X	X
UMP	-5.75	2.56	2.66	0.10	0.09	1.02	2.00	2.12	23.64	23.53	-0.11	0.02	0.07	12.21	12.15
zeatin	0.06	12.76	12.74	-0.02	0.48	0.08	13.96	13.93	16.75	6.77	-9.98	0.99	2.41	8.36	2.78
giberellic acid	-0.37	19.78	19.73	-0.05	0.31	0.08	22.18	22.13	3.28	3.30	0.02	1.17	0.35	0.83	0.84
jasmonic acid	2.28	27.22	27.16	-0.06	0.12	0.04	30.90	30.83	3.00	3.00	0.01	0.33	0.02	0.67	0.68
abscisic acid	1.90	24.85	24.78	-0.07	0.25	0.12	28.12	28.04	3.09	3.09	0.00	0.32	0.38	0.73	0.73
linoleic acid	7.08	23.57	23.57	-0.01	0.15	0.12	26.63	26.62	2.93	3.27	0.35	0.31	1.21	0.64	0.83
epibrassinolide	3.46	29.18	29.21	0.04	0.04	0.06	33.19	33.24	3.22	3.27	0.06	1.53	0.68	0.80	0.83
spermidine	-6.99	1.65	1.88	0.24	0.07	0.55	0.93	1.21	30.04	30.89	0.85	0.17	0.08	15.78	16.26
chloritamic acid	-0.43	20.63	20.65	0.02	0.07	0.08	23.17	23.20	3.29	3.41	0.12	1.81	1.62	0.84	0.90
coumaric acid	1.50	20.37	20.27	-0.10	0.45	0.08	22.87	22.75	3.21	2.82	-0.39	1.21	1.52	0.79	0.58
				1.61	9.63	18.52	240.36	242.25			-14.23	13.27	18.31	167.26	156.31

Table1 : logD (calculated with Pallas 3.0®); mean t_R, difference in t_R, RSD% and capacity factor for each metabolite in pure sample and extract (positive ion mode; n=3) on the Atlantis dC18 column and the TSKgel Amide-80 column.

❖The retention time reproducibility of the HILIC column can be compared with that of the Atlantis dC18 column, as seen in *Table 1*. The retention time shift of zeatin on the TSKgel Amide-80 column is conspicuous. This together with the bad peak shape for some compounds (see *Figure 1*) results in the conclusion that the hydrophilic interaction column provides data less suitable for metabolomics data processing software, as these data mining programs bunch mass with retention time in complex LC-MS matrices.

❖Hydrophilic interaction chromatography, nevertheless provides increased peak areas for the most polar compounds compared to the reversed-phase column.

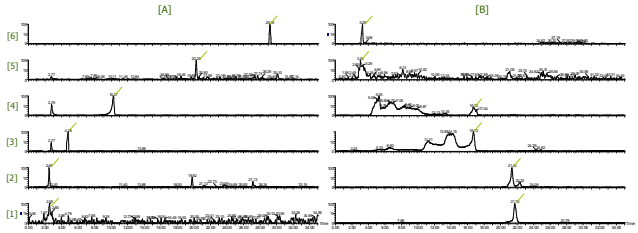


Figure 1: Mass chromatograms of [1] serine (*m/z* 106), [2] lactose (Na^+, *m/z* 365), [3] adenine (*m/z* 136), [4] phenylalanine (*m/z* 166), [5] coumaric acid (*m/z* 165) and [6] epibrassinolide (*m/z* 481) on [A] the Atlantis dC18 column and [B] the TSKgel Amide-80 column, in positive ion mode

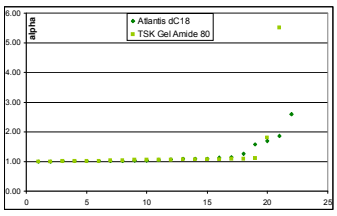


Figure 2: Selectivity factors in rising order of magnitude for both columns.

❖In comparing the chromatographic performance, the reversed-phase column definitely performs better in terms of peak capacity factor (*Table 1*). The selectivity of both columns is comparable, as seen in *Figure 2*. The Atlantis dC18 column only shows a steeper rise in selectivity factors. This indicates that the reversed-phase provides better selectivity for the compounds it is, in essence, most suited for (apolar compounds) than the HILIC for its major application field (polar compounds).

Conclusion

❖In metabolomics analysis, many techniques have to be joined to obtain a complete image of the metabolome. In this perspective, hydrophilic interaction chromatography can have some added value, especially for the analysis of more polar compounds.

❖In terms of data processing, one has to be aware that good peak shape and reproducibility of retention times is crucial. According to this criterion, we are less inclined to choose for the TSKgel Amide-80 column. This is due to bad peak retention capacities for several metabolites compared to a reference reversed-phase column.

LC-MS based metabolomics and accurate mass measurements in complex extracts

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Introduction

Metabolomics represents the in-depth profiling of metabolites intrinsic to several organisms. An important feature of metabolomics is identifying differences in metabolite patterns, thus demanding accurate measurements in analytical devices. Here we discuss accurate mass measurement on a Q-TOF micro (Waters) equipped with a Lockspray device.

Aim

This part of our research investigates the possibility of accurate mass measurements in a metabolomic context. Our goal was to establish the mass accuracy of a Q-TOF micro (Waters), using the features available in the Masslynx software. A comparison was made between leucine-enkephalin as lockmass, and a mixture of compounds that covers the mass range 50-800.

Materials

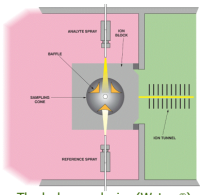
HPLC Conditions:
- Alliance 2695 (Waters®)
- Column:
  - Atlantis™ dC18, 2.1 x 150mm (Waters®)
- Mobile Phase:
  - Eluent A: 100% water + 0.1% formic acid
  - Eluent B: 90/10 acetonitrile/water + 0.1% formic acid
- Flow Rate: 200µl/min
- Injection Volume: 50µl

MS Conditions:
- Mass Spectrometer: Q-TOF micro (Waters®)
- Ion Source: Lockspray™ in positive ion mode
- Software: Masslynx 4.0
- *m/z*-range: 50-800
- Capillary Voltage: 3000V
- Cone Voltage: 30V

Metabolites:
Amino acids, carbohydrates, nucleotides, plant hormones, steroids, fatty acids, polyamines, carotenoids and others were used from stock solutions at 1mg/ml

Lockspray solution:
- Leucine-enkephalin 1ng/µl in 50/50 AcN/H_2O
  m/z-value: 556.2771
- Lockmix:
  - Acetanilide (*m/z* 136.0762)
  - Pentazocine (*m/z* 286.2171)
  - Trazodone (*m/z* 374.1747)
  - Dipyridamole (*m/z* 505.3251)
  - Aconitine (*m/z* 646.3227)

Extraction procedure:
Arabidopsis thaliana leaves were homogenized with an Ultra-Turrax mixer (Ika) in 2:6:2 H_2O:MeOH:$CHCl_3$. Samples were centrifuged for 10 minutes and the supernatant (after spiking) was evaporated under nitrogen. Before injection, samples were dissolved in 75/25 H_2O/AcN + 0.1% FA.



The lockspray device (Waters®)

Methods

❖A mixture of 5 compounds with lockmasses covering the typical mass range in metabolomic research from 50-800, is considered to give better accurate mass measurements than a single compound. Leucine-enkephalin is the standard product in accurate mass measurement using lockspray devices. Both lockspray compositions were tested to evaluate the best accurate mass measurement.

❖Accurate mass measurement is an important feature in identifying metabolites in metabolomics. Subtle differences from multivariate data analysis can thus be attributed to specific metabolites, revealing complex biochemical regulations in e.g. plants.

Results

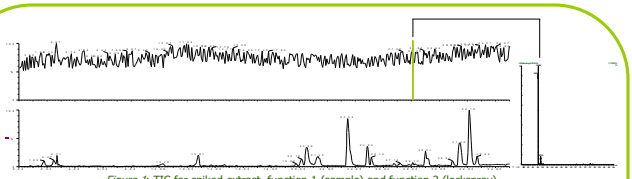


Figure 1: TIC for spiked extract, function 1 (sample) and function 2 (lockspray), using leucine-enkephalin as a lockmass; spectrum of leu-enk.

Name compound	Accurate mass (Da)	Mass accuracy (ppm; n=3)
leucine	132.1025	8.83 ± 1.58
phenylalanine	166.0868	11.24 ± 2.28
s-adenosylmethionine	399.1451	-2.92 ± 4.00
lactose	365.106	-4.93 ± 8.55
maltotriose (Na+)	527.1588	2.85 ± 1.71
uracil	113.0351	3.24 ± 5.03
cytidine	244.0933	2.59 ± 12.31
IMP	349.0594	6.11 ± 5.45
zeatin	220.1198	-3.03 ± 2.15
jasmonic acid	211.1334	-3.16 ± 7.28
abscisic acid	265.144	0.00 ± 5.07
epibrassinolide	481.3529	1.94 ± 3.72
spermidine	146.1657	-0.23 ± 3.77

Table 1: Several spiked metabolites with accurate mass (Da) and mass accuracy in ppm, using leucine-enkephalin as a lockmass (n=3)

Leucine-enkephalin (*Figure 1*) is presented as lockmass in accurate mass measurement. By means of the accurate mass measure tool in Masslynx, *m/z* correction throughout the entire chromatogram is possible. When analysing complex LC-MS matrices, data processing software bunches mass with retention time. In this perspective, masses are taken at most intense peak signal. Mass deviations up to 40ppm are obtained (results not shown). Such deviations are too large for identification purposes but adequate for multivariate data analysis where binning of masses is the rule. When mass correction is performed at a signal intensity (e.g. at the foothill of a peak) close to the intensity of the lockmass, however, mass differences lay within 10ppm (*Table 1*).

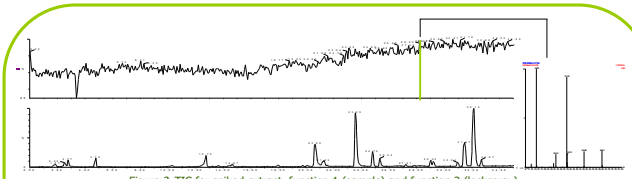


Figure 2: TIC for spiked extract, function 1 (sample) and function 2 (lockspray), using the lockmix for accurate mass measurement; spectrum of lockmix.

Name compound	Accurate mass (Da)	Mass accuracy (ppm; n=3)
leucine	132.1025	42.39 ± 9.66
phenylalanine	166.0868	45.96 ± 27.76
s-adenosylmethionine	399.1451	38.00 ± 16.20
lactose	365.106	37.07 ± 6.17
maltotriose (Na+)	527.1588	-1.83 ± 2.08
uracil	113.0351	49.24 ± 12.89
cytidine	244.0933	-36.87 ± 26.31
IMP	349.0594	11.27 ± 7.04
zeatin	220.1198	9.24 ± 3.22
jasmonic acid	211.1334	13.74 ± 5.76
abscisic acid	265.144	-2.39 ± 10.05
epibrassinolide	481.3529	16.20 ± 2.40
spermidine	146.1657	41.28 ± 9.29

Table 2: Several spiked metabolites with accurate mass (Da) and mass accuracy in ppm, using the lockmix for accurate mass measurements (n=3)

Via a tool in Masslynx, called secondary reference correction, a possibility is provided to correct masses using a mixture of compounds. Here we present a lockmix of 5 compounds covering the *m/z* range 50-800 (*Figure 2*). The secondary reference tool corrects masses with the lockmass closest to the *m/z* of the sample metabolite. Theoretically, better lockmass correction could be envisaged. Nevertheless, larger deviations are observed as can be seen in *Table 2*.

Conclusion

❖We tried to obtain a better lockmass-correction in the *m/z* range 50-800 using a mixture of 5 compounds. Nevertheless, leucine-enkephalin seems the best solution for *m/z*-correction in a lockspray setting.

❖Accurate mass measurements with a Q-TOF micro are feasible within a mass range of 10ppm, if the signal intensity of compound and lockspray are matched.