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4 Simultaneous analysis of folic acid and pantothenic acid  
5 in foods enriched with vitamins by stable isotope dilution  
6 assays

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## 1 ABSTRACT

2 Folic and pantothenic acid were quantified in multivitamin products by stable isotope  
3 dilution assays using [<sup>2</sup>H<sub>4</sub>]-folic acid and [<sup>13</sup>C<sub>3</sub>, <sup>15</sup>N]-pantothenic acid as the internal  
4 standards. Detection was achieved by liquid chromatography-mass spectrometry  
5 which enabled unequivocal determination of the vitamins. Due to the very simple  
6 extraction procedure, analysis of the vitamins was completed within two hours. When  
7 analyzing multivitamin sweets, the intra-assay and inter-assay coefficient of variation  
8 was 3.2 % (n=5) and 3.1 % (n=5) for folic acid and 4.5 % (n=5) as well as 6.5 %  
9 (n=7) for pantothenic acid, respectively. Along with the precision data, recovery  
10 values of 99.4 % for folic acid and 103 % for pantothenic acid at addition levels of 6  
11 mg/kg and 600 µg/kg, respectively, to starch products proved the accuracy of the  
12 new method. Application of the stable isotope dilution assay to fruit juices, whey  
13 products, cereals, sweets, pharmaceuticals, wheat flour and salt fortified with one or  
14 both vitamins revealed that for the majority of products the labelled pantothenic acid  
15 contents were exceeded by about 30 %, whereas for folic acid also significantly lower  
16 contents than the label claim were found.

17

18 *Key words:* folic acid, liquid chromatography – tandem mass

19 spectrometry, multivitamin products; pantothenic acid, stable isotope

20 dilution assay

21

## 1 INTRODUCTION

2 In the nutrition sciences evidence is accumulating that vitamins are not only essential  
3 for maintaining normal physiological functions but also to prevent from hazards  
4 resulting from oxidative stress or disorders of cell division and DNA repair. In  
5 particular the vitamins of the folate group are supposed to prevent neural tube  
6 defects, Alzheimer's disease and colon cancer. Most recently, a review highlighted  
7 that folate deficiency may induce single strand breaks and mimic radiation damages  
8 [1]. Therefore, a lot of foods are supplemented with vitamins and for cereal products,  
9 fortification with folic acid is mandatory in the USA. Analysis is commonly performed  
10 for each vitamin separately, apart from some HPLC applications to pharmaceutical  
11 multivitamin products. As pharmaceuticals contain vitamins in the mg/g range, the  
12 universal UV detection is sufficient to analyze water-soluble as well as fat-soluble  
13 vitamins [2-4]. However, in fortified foods vitamin levels are much lower, thus  
14 rendering UV detection too insensitive for a couple of compounds. For instance,  
15 pantothenic acid (vitamin B<sub>5</sub>, PA) contains no chromophore and, therefore, is often  
16 obscured by matrix interferences as it has to be detected at 200 nm. Fluorescence  
17 detection, however, although being more selective and sensitive, is restricted to  
18 thiamine, riboflavin, pyridoxine and not feasible to detect PA, folic acid, and niacin.  
19 By contrast, mass spectrometry (MS) coupled to liquid chromatography (LC) appears  
20 to be suited to detect selectively, sensitively and universally most of the vitamins.  
21 However, as ion yields in common atmospheric pressure ionization sources show  
22 wide dispersions within one single chromatographic run, the use of internal standards  
23 is essential. As we recently developed stable isotope dilution assays (SIDA) for  
24 pantothenic acid via GC/MS [5] and for folates via LC/MS/MS detection [6], it was the  
25 goal of the current study to combine these methods into a preliminary multivitamin

1 method. Since SIDAs exhibit major specificity, accuracy, and sensitivity, this approach  
2 offers the perspective for a candidate reference method.

### 3 EXPERIMENTAL

#### 4 Chemicals

5 The following chemicals were obtained commercially from the sources given in  
6 parentheses: (R)-pantothenic acid hemicalcium salt (Aldrich, Steinheim, Germany);  
7 acetonitrile, formic acid, hydrochloric acid,  $\text{KHCO}_3$ , methanol,  $\text{Na HCO}_3$ , sodium  
8 acetate,  $\text{Na}_2\text{SO}_4$ , (Merck, Darmstadt, Germany).

9  $[\text{}^{15}\text{N}, \text{}^{13}\text{C}_3]$ -pantothenic acid and  $[\text{}^2\text{H}_4]$ -folic acid were synthesized as reported recently  
10 [5, 7].

11 Extraction buffer consisted of aqueous HEPES (50 mmol/L) and aqueous CHES (50  
12 mmol/L) at pH 7.85 and contained sodium ascorbate (2 %) and 2-mercapto ethanol  
13 (20 mM).

14

#### 15 Stable isotope dilution assay (SIDA) for the determination of free PA in 16 foods

17 Tablets and sweets were ground in a mortar and breakfast cereals in a grain mill  
18 (Bosch, München, Germany). The resulting powders or flours (0.5 g) were stirred for  
19 one hour at 20 °C in extraction buffer containing calcium  $[\text{}^{15}\text{N}, \text{}^{13}\text{C}_3]$ -(R)-pantothenate  
20 (10 µg) and  $[\text{}^2\text{H}_4]$ -folic acid (400 ng). To juices and whey products the labeled  
21 standards were added directly.

22 The extracts were filtered and, after passing through an syringe filter (0.4 µm,

1 Millipore, Bedford, MA, USA), analyzed by LC/MS/MS.

2

### 3 Liquid chromatography / mass spectrometry (LC/MS)

4 LC/MS and LC/MS/MS was performed by means of an LCQ (Finnigan MAT, Bremen,  
5 Germany) coupled to a spectra series high performance liquid chromatograph  
6 (Thermo separation products, San Jose, CA, USA) equipped with an Aqua C-18  
7 reversed phase column (250 x 4.6 mm; 5  $\mu$ m, Phenomenex, Aschaffenburg,  
8 Germany). 50  $\mu$ L of the sample solutions were chromatographed using gradient  
9 elution with variable mixtures of aqueous formic acid (0.1 %, solvent A) and  
10 acetonitrile (solvent B), at a flow of 0.8 mL/min. After flushing the column for 9 min  
11 with 7% B, a 13-min linear gradient was programmed to 17 % B followed by a further  
12 3-min linear gradient to 25 % B. Then, the concentration of B was raised immediately  
13 to 100 %, maintained for 5 min and subsequently brought back to the initial mixture  
14 for another 5 min to allow for column equilibration. During the first 8 min of the  
15 gradient programme, the column effluent was diverted to waste to ensure an  
16 adequate spray stability. For LC/MS/MS of pantothenic acid, the mass transitions  
17 ( $m/z$  precursor ion/  $m/z$  product ion) 224/206 and 220/202 for labeled and unlabelled  
18 pantothenic acid, respectively, were chosen. For folic acid, the mass transitions  
19 446/299 and 442/295 were recorded for the labeled and unlabelled isotopomer,  
20 respectively. The isolation width of the precursor ion was adjusted to 3 Da and the  
21 isolation width of the product ion was set to 1 Da in order to detect the product ion  
22 most selectively. The mass spectrometer operated in the positive electrospray mode  
23 with a spray needle voltage of +5 kV and a spray current of 20  $\mu$ A. The temperature  
24 of the capillary was 200° C and the capillary voltage was +13 V. The sheath and

1 auxillary gas nitrogen nebulized the effluent with flows of 68 and 19 arbitrary units,  
2 respectively. The ion trap was operated at a helium pressure of  $10^{-3}$  Torr.

3

#### 4 Determination of response factors for LC-MS/MS

5 Solutions of calcium pantothenate/ calcium [ $^{15}\text{N}$ ,  $^{13}\text{C}_3$ ]-pantothenate as well as folic  
6 acid / [ $^2\text{H}_4$ ]-folic acid in extraction buffer were mixed in nine mass ratios ranging from  
7 0.1 to 9 to give a total volume of 10 mL. Subsequently, the mixtures were subjected  
8 to LC/MS/MS as outlined before. Response factors  $R_f$  were calculated as reported  
9 recently [5].

10

#### 11 Determination of detection and quantification limits

12 30, 110, 220 and 560 ng of PA were added to edible corn starch (1g) and analyzed  
13 as detailed before. Each sample was analyzed in triplicates. Detection (DL) and  
14 quantification limits (QL) were calculated according to Hädrich and Vogelgesang [7]:  
15 DL is the concentration calculated from the maximum height of the 95 % confidence  
16 interval at the zero addition level. QL is the addition level for which the lower 95 %  
17 confidence limit equals the upper 95 % confidence limit of the addition level at the  
18 DL. QL and DL of folic acid were determined as reported recently [6].

19

## 1 PRECISION AND RECOVERY

2 Intra-assay precision was evaluated by analyzing a multivitamin sweet as detailed  
3 before (n=5).

4 Recovery was determined by adding 6 µg of PA and 600 ng of FA to edible corn  
5 starch (1g) and performing SIDA as detailed before in quadruplicate analysis.

6

## 7 RESULTS AND DISCUSSION

### 8 LC/MS of pantothenic acid and folic acid

9 Recently we reported on two SIDAs, the first to quantify PA by GC/MS-detection [5]  
10 and the second to analyze folates by LC-tandem MS [6]. Both assays were based on  
11 the use of isotopomeric vitamins as the internal standards. As PA is a very polar  
12 molecule, it appeared to be also detectable sensitively by electrospray ionization  
13 (ESI)-MS. This assumption was confirmed by preliminary experiments on aqueous  
14 solutions of PA, which revealed a conceivable signal of the quasimolecular ion at  $m/z$   
15 220 in positive ESI-MS as depicted in fig. 1.

16 Besides  $[M + H]^+$  two minor signals at  $m/z$  242 and 461 appeared, which can be  
17 assigned to  $[M + Na]^+$  and  $[2M + Na]^+$ , respectively. Analogously,  $[^{15}N, ^{13}C_3]$ -PA gave  
18 signals at  $m/z$  224, 246 and 469.

19 As the recently developed SIDA of folates was based on LC/MS/MS due to matrix  
20 interferences [6], tandem MS was also applied to PA isotopomers in case of being  
21 necessary for unequivocal quantification. By employing collision-induced dissociation  
22 (CID) to the protonated molecule of isotopomeric PA, the spectrum shown in fig. 2

1 was obtained. Subsequent experiments revealed that the signal at  $[M-18]^+$  could be  
2 used for differentiation and quantification of the isotopomers.

3 The calibrating curves of different ratios of the isotopomers revealed linearity for the  
4  $[M + H]^+$  in single stage and for the product ion  $[M-18]^+$  of the protonated molecule in  
5 MS/MS over two decades of isotope ratios.

6 For simultaneous LC of PA and folic acid (FA), the system for folates [Freisleben et  
7 al., 2003] was adopted. LC/MS in single stage MS of a cereal extract revealed a  
8 good peak shape for PA isotopomers, whereas the mass chromatograms of FA and  
9  $[^2H_4]$ -FA contained several matrix interferences as shown in fig 3. Therefore, tandem  
10 MS was applied, which improved significantly specificity and peak shapes as displayed  
11 in fig 4 .

## 12 Extraction and analysis of free folic acid and free pantothenic acid

13 The extraction buffer according to Wilson and Horne [8], which proved to be most  
14 effective for folates, was also best suited for extraction of PA. Therefore, sample  
15 preparation for LC/MS of PA and FA proved to be very simple. After stirring the  
16 powdered samples for 1 h in extraction buffer containing known amounts of  
17  $[^{15}N, ^{13}C_3]$ -PA and  $[^2H_4]$ -folic acid at pH 5.7, the extracts only had to be filtered and  
18 passed through a membrane filter. Detection was achieved either by single stage or  
19 tandem MS. In contrast to non-fortified foods, most of the samples analyzed here  
20 contained only minute amounts of conjugated vitamins. Therefore, enzymatic  
21 liberation, on the one hand, of bound PA by phosphatase and pantothenase [5] and,  
22 on the other hand, of bound FA by amylase, proteinase and deconjugase [6] was  
23 evitable.



## 1 Performance criteria

2 To evaluate whether sensitivity of LC-MS was sufficient for quantifying PA in foods,  
3 the detection limit (DL) was determined in edible starch according to the method of  
4 Hädrich and Vogelgesang [9].

5 The calculations resulted in a DL of 89  $\mu\text{g}/100\text{g}$  and a quantification limit (QL) of 200  
6  $\mu\text{g}/\text{kg}$  for PA and a DL of 40  $\mu\text{g}/\text{kg}$  for FA in starch containing foods. Recoveries were  
7 evaluated by adding 6 mg/kg of PA and 600  $\mu\text{g}/\text{kg}$  of FA to edible starch and were  
8 found to be 99.4 and 103 %, respectively.

9 For examining precision, a multivitamin sweet was analyzed and revealed an intra-  
10 assay and inter-assay coefficient of variation of 3.2 % and 3.1 %, respectively. All  
11 performance data mentioned before are summarized in table 1.

12

## 13 Results of the quantifications of foods

14 Several foods fortified by several vitamins were surveyed to prove the suitability of  
15 the new method. Of liquid products, four fortified fruit juices and two whey products  
16 were quantified. Moreover, two samples of breakfast cereals, two sweets and one  
17 multivitamin pharmaceutical were analyzed. Finally we quantified a meal for weight  
18 reduction, a breakfast drink as well as a wheat flour and salt, the latter of which were  
19 solely fortified with folic acid. The results of the quantifications are presented in table  
20 2.

21 Regarding PA, the contents ranged from 0.39 / 100g in the wheat flour to 125 mg  
22 /100g in the pharmaceutical. In nearly all products the PA label claim was exceeded

1 by 10 to 50 %, except one fruit juice, which contained only 60 % , one cereal  
2 containing 240 % and one whey containing 225 % of the amounts declared on the  
3 label. As already reported by Romera et al. [10] on infant formulas, overfortifications  
4 by up to 260% of the PA label claim are common, which was confirmed by our data.  
5 In contrast, the PA content of the pharmaceutical was exactly the labelled one.  
6 Considering folic acid, the contents ranged between 20 and 8380 µg/100g. Contrary  
7 to PA, the folate contents were much more scattered and exceeded only for 7  
8 products the label claim, whereas 5 products contained significantly lower amounts  
9 than labelled. Only two fruit juices and the wheat flour were well in line with the label  
10 within a tolerance of 10 %. Remarkable discrepancies were found on the one hand  
11 in case of the breakfast cereals, which exceeded the label claim by more than 50 %  
12 for both vitamins. Although an overdosage is thought to be reasonable to anticipate  
13 losses during manufacture and storage, these differences to the label appear too  
14 high. On the other hand, the breakfast drink and the meal for weight reduction both  
15 did not reach the labelled FA content, which was first suspected to be due to  
16 endogenous food folates stemming from the single ingredients. To prove this  
17 assumption, the folates were analyzed by the SIDA reported recently [6]. However, in  
18 both products only about 6 µg/100g folates different from folic acid were found, which  
19 did not significantly contribute to the total folate content. The LC-mass  
20 chromatogramms of the slim meal extract is displayed in fig 3.

21 These findings are in quite accordance with the results of Osseyi et al. [11] who  
22 analyzed folic acid in fortified cereal products by LC-UV. However, the data of the  
23 latter authors ranged between 72 and 147 % of the label claim and thus showed  
24 lower discrepancies than the products analyzed in the present study.

25

26

## 1 CONCLUSION

2

3 In particular folic acid is a common vitamin for food fortification in order to prevent the  
4 aforementioned disorders. Considering the recommended intakes of 400 µg/d and  
5 the upper limit of 1000 µg/d [12], consumers have to rely on the labelled content to  
6 make their diet meet the recommendations. However, our results indicate, that  
7 differences for FA up to 110 % above and 20 % below the label claim occur in  
8 multivitamin products. Therefore, the manufacturers are called upon adjusting the  
9 folate contents more accurately and the official laboratories upon controlling the  
10 contents more frequently.

11 The method presented here reveals excellent accuracy and sensitivity. Other water-  
12 soluble vitamins such as pyridoxine or niacin contain nitrogen and, therefore, should  
13 be ionizable and detectable by LC/MS. This offers the perspective to include these  
14 vitamins into the method presented here and to open inroads into a multivitamin  
15 SIDA by LC/MS, which would enable simultaneous, fast and accurate quantification  
16 of vitamins.

## 17 ACKNOWLEDGEMENT

18 I am grateful to Mrs. D. Fottner for her excellent technical assistance. Appreciation is  
19 also due to Mrs. I. Otte for running the LC/MS equipment.

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12  
13

1 **Table 1.** Comparison of performance data of the stable isotope dilution assays  
 2 (SIDA) based on LC/MS/MS to that based on GC/MS

Performance criterion	Folic acid	Pantothenic acid
Detection limit	40 µg/kg	86 µg/kg
Quantification limit	120 µg/kg	240 µg/kg
intra-assay CV	3.2 % (n=5)	4.5 % (n=5)
inter-assay CV	3.1 % (n=5)	6.5 % (n=7)
in multivitamin sweets		
Recovery	99.4 % ± 2.0 % (n=3)	103.0 ± 6.5% (n=4)
	Addition level: 6 mg/kg	Addition level: 0.6 mg/kg

18 n.d. not determined; CV coefficient of variation

19

20

1 **Table 2.** Analyzed and labelled contents of pantothenic acid and folic acid in foods

2

mg/100g Sample	Panto thenic acid			Folic acid		
	analyzed	label claim	% of claim	analyzed	label claim	% of claim
Fortified Fruit juices	3.58	2.50	143%	0.24	0.20	120%
	2.66	2.00	133%	0.07	0.07	103%
	3.79	3.00	126%	0.12	0.10	116%
	1.86	3.00	62%	0.09	0.10	94%
Whey products	2.03	0.90	225%	0.04	0.03	133%
	1.35	1.20	112%	0.05	0.04	131%
Breakfast Cereals	7.94	5.10	156%	0.27	0.17	161%
	14.65	6.00	244%	0.43	0.20	213%
Sweets	23.31	18.00	130%	0.7	0.60	117%
	36.10	29.70	122%	0.57	0.80	72%
Pharma- ceutical	115.1	115.00	100%	2.96	3.85	77%
Breakfast drink	0.91	0.60	152%	0.02 <sup>a</sup>	0.06	37%
Meal for weight reduction	0.84	0.65	130%	0.02 <sup>b</sup>	0.02	87%
Fortified wheat flour	0.36	-	-	0.14	0.14	103%
Fortified salt	n.d.	-	-	8.38	10	84%

3

4 n.d. not detectable

5 <sup>a</sup> containing 6.1 µg/100g endogenous food folates6 <sup>b</sup> containing 6.4 µg/100g endogenous food folates

7

## 1 Legends to the Figures

2 **Figure 1.** Mass spectrum of pantothenic acid (above) and [ $^{15}\text{N}$ ,  $^{13}\text{C}_3$ ]-pantothenic  
3 acid (below) in positive electrospray ionization mode.

4 **Figure 2.** MS/MS spectrum of pantothenic acid (above) and [ $^{15}\text{N}$ ,  $^{13}\text{C}_3$ ]-pantothenic  
5 acid (below) after collision-induced dissociation (CID) of the  
6 quasimolecular ions in positive electrospray ionization mode.

7 **Figure 3.** Single stage mass chromatograms of fortified breakfast cereals containing  
8 7.94 mg/100g of pantothenic acid (PA) and 270  $\mu\text{g}$  / 100g of folic acid  
9 (FA).

10 **Figure 4.** MS/MS chromatograms of fortified breakfast cereals containing 7.94  
11 mg/100g of pantothenic acid (PA) and 270  $\mu\text{g}$  / 100g of folic acid (FA).

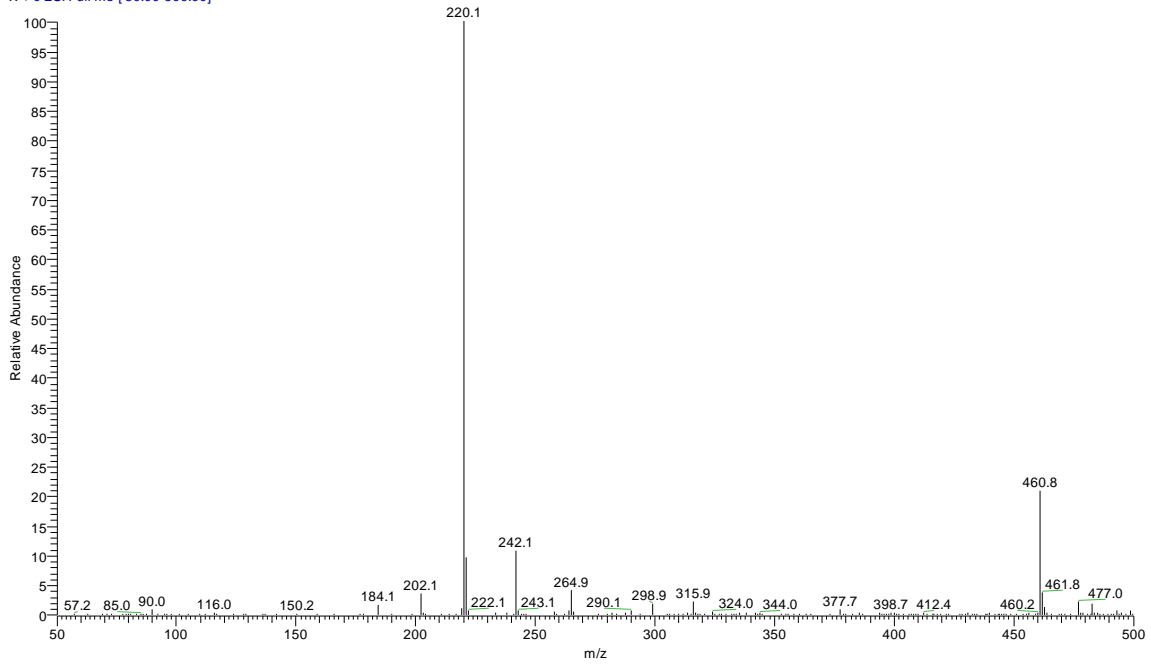
12 **Figure 5.** MS/MS chromatograms of a breakfast drink containing 2.1  $\mu\text{g}$ , 3.2  $\mu\text{g}$ , and  
13 14  $\mu\text{g}$  / 100 g of 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, and  
14 folic acid, respectively.

15

16

1

2-281102 #1127-1149 RT: 15.75-16.07 AV: 23 SB: 31 16.88-17.33 NL: 3.67E6  
T: + c ESI Full ms [50.00-500.00]

2  
3

1-281102\_021128151528 #1260-1283 RT: 17.73-18.06 AV: 24 SB: 30 18.50-18.93 NL: 5.45E6  
T: + c ESI Full ms [50.00-500.00]

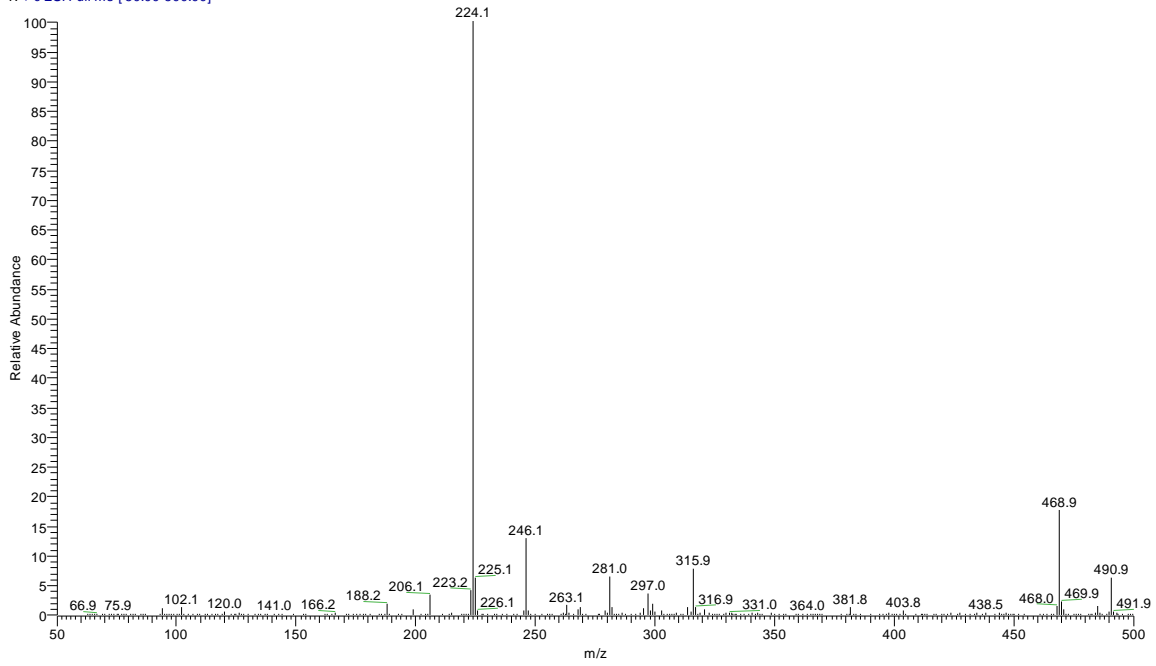
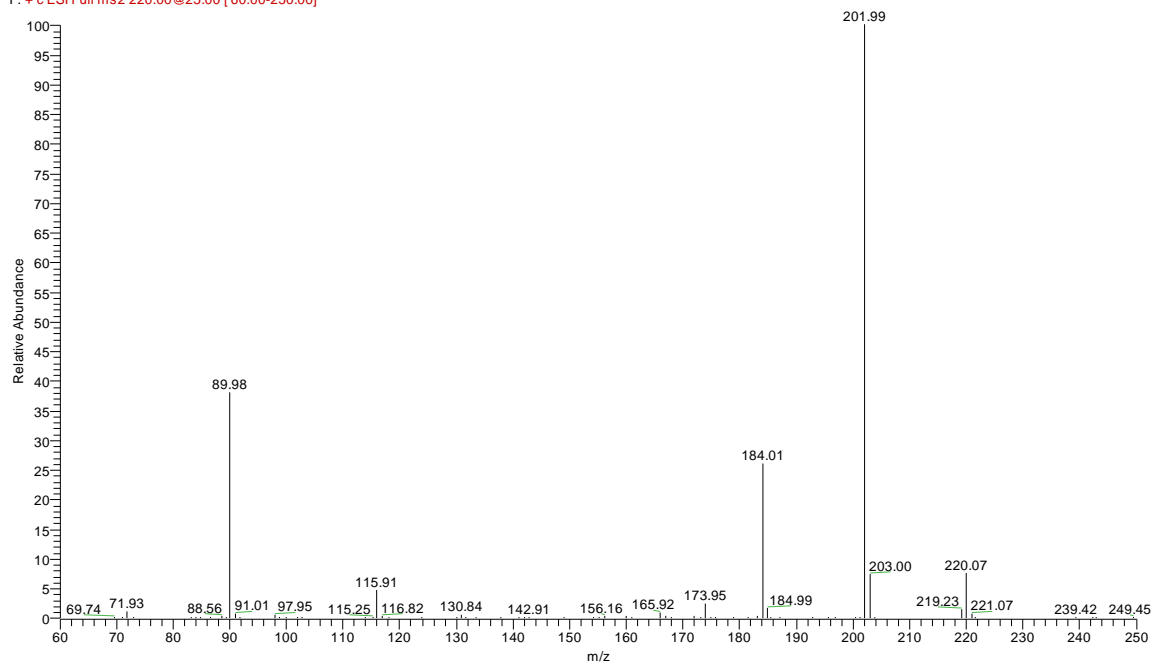
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fig. 1

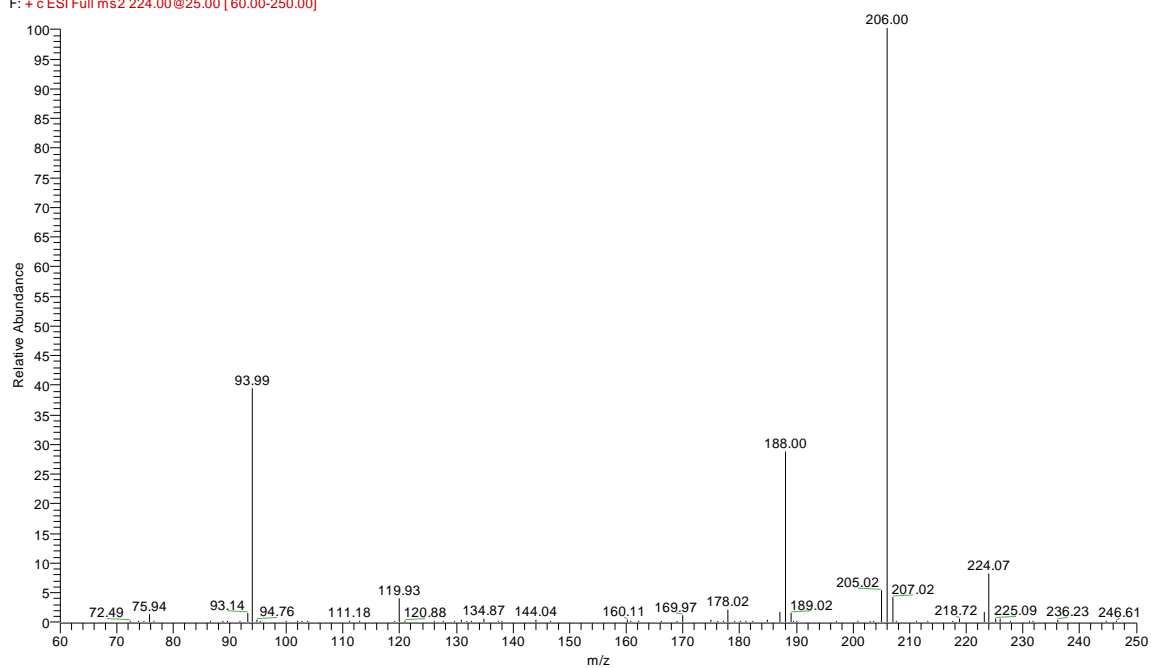


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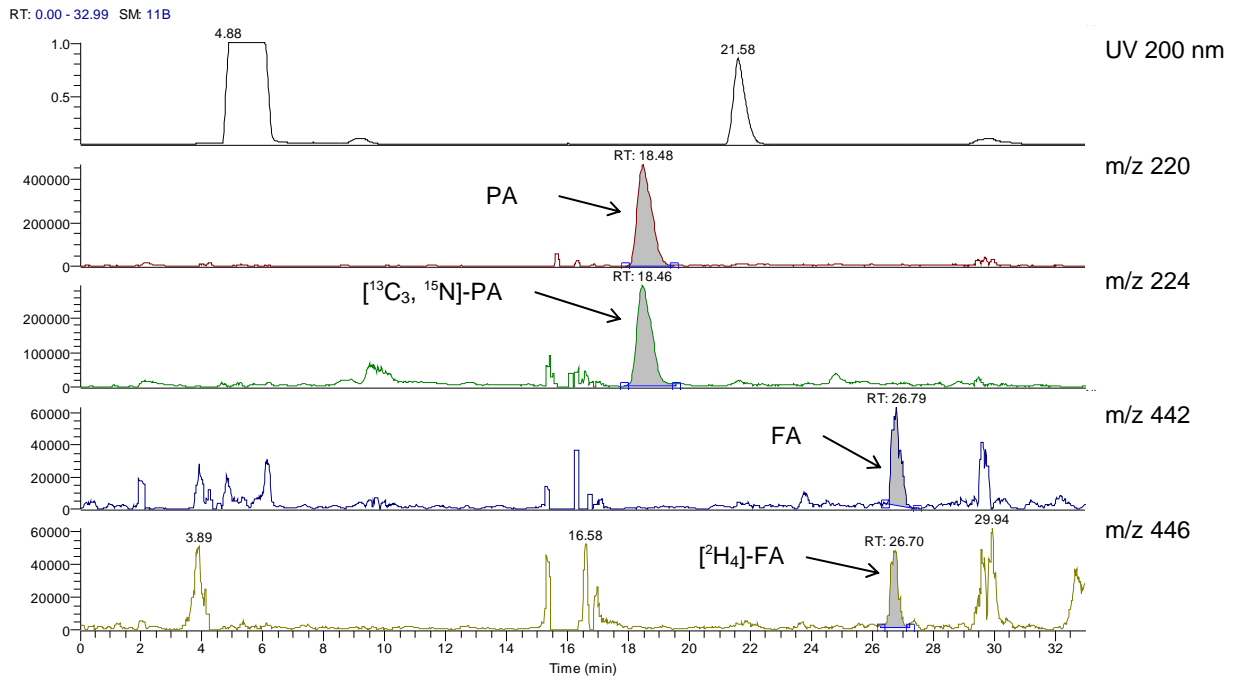
1  
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3

2-171002\_021017140319 #861-884 RT: 16.94-17.36 AV: 12 SB: 14 17.81-18.36 NL: 3.23E5  
F: + c ESI Full ms2 224.00@25.00 [ 60.00-250.00]



4  
5  
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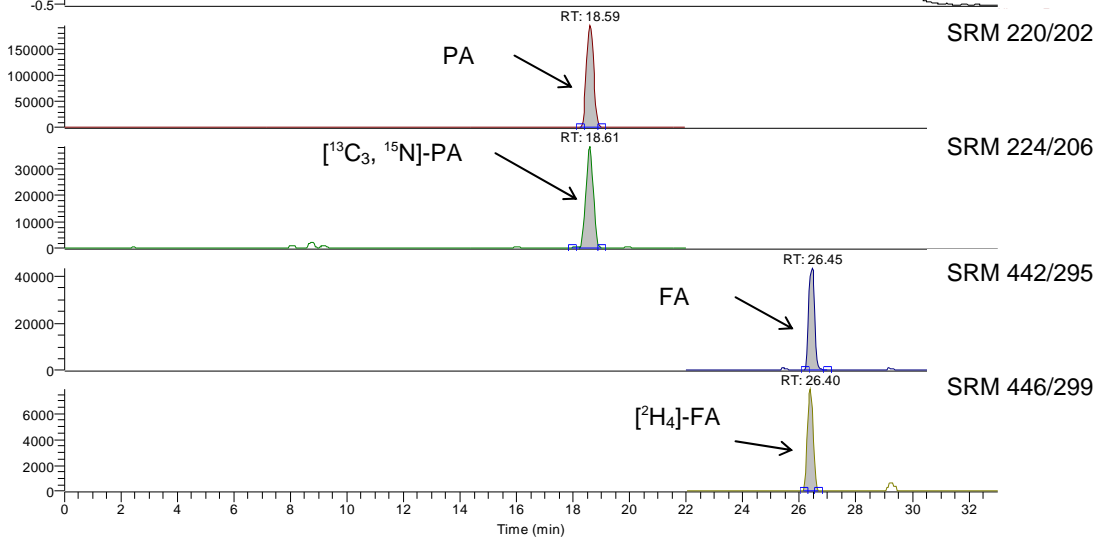
fig. 2



1  
2  
3  
4

RT: 0.00 - 32.99 SM: 7B

UV 200 nm



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3  
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