



Sphinganine and sphingosine levels and ratio in urine and blood samples from a Catalonian population (Spain).

Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2010-394.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	28-Feb-2011
Complete List of Authors:	Cano-Sancho, German; University of Lleida, Food Technology Marín, Sonia; University of Lleida, Food Technology Sanchis, Vicent; University of Lleida, Food Technology Colom, Carme; University of Lleida, Food Technology Coronel, Maria Bernarda; University of Lleida, Food Technology Ramos, Antonio; University of Lleida, Food Technology
Methods/Techniques:	Chromatography - HPLC, Exposure assessment
Additives/Contaminants:	Fumonisin
Food Types:	Beer, Cereals
Abstract:	<p>Fumonisin occurs mainly in maize, and produces alterations in sphingolipid metabolism, unbalancing sphinganine (Sa) / sphingosine (So) ratio. This alteration has been proposed as a biomarker of fumonisin exposure.</p> <p>The objective of the present work was to study the urinary and plasmatic levels of Sa, So as well as, the ratio Sa/So from a sample of Catalonian population exposed at low levels of fumonisin. Firstly, plasmatic and urinary Sa and So levels and the ratio Sa/So were compared between two population groups, and after, urinary Sa and So levels from corn-food consumers and a control group were monitored during two weeks under controlled intake of corn-foods. Sa and So levels were determined in urine and blood samples using validated methods using HPLC with fluorescence detection.</p> <p>Significant differences were not found in urine samples when Sa/So ratios were compared from corn-food consumers and non</p>

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

	<p>consumers, while significant differences were found in urine and plasma samples but evidences of mechanism of action of fumonisins were not apparent. Through time-course study, we have narrowed down the day in which the maximum alteration of Sa/So ratio should be expected in humans. In this paper we have reported some useful information to improve the design of studies to validate the ratio Sa/So as a possible biomarker of fumonisin exposure.</p>

For Peer Review Only

SCHOLARONE™
Manuscripts

1
2
3 **Sphinganine and sphingosine levels and ratio in urine and blood**
4 **samples from a Catalonian population (Spain).**
5
6
7
8
9

10
11 G. CANO-SANCHO^{a*}, S. MARIN^a, V. SANCHIS^a, C. COLOM^a, M.B. CORONEL^a and
12
13 A.J. RAMOS^a.
14
15

16
17
18 *Corresponding author: German Cano-Sancho. Food Technology Department, University
19 of Lleida, Rovira Roure 191, 25198, Lleida. Telephone number: (+0034) 973702535. Fax
20 number: (+0034) 973702596. E-mail address: gcano@tecal.udl.cat
21
22
23
24
25

26
27
28 ¹Food Technology Department, University of Lleida, XaRTA-UTPV, Lleida, Spain;
29
30 Rovira Roure 191, 25198.
31
32

33
34
35 Keywords: fumonisin; biomarker; sphinganine; sphingosine; urine; blood.
36
37

38
39 Abbreviations: Enzyme Ceramide (CER), Food and Agriculture Organization (FAO),
40 Food and Drug Administration (FDA), food frequency questionnaire (FFQ), fumonisin
41 B₁(FB₁), fumonisin B₂ (FB₂), total fumonisins (FB_T), high performance liquid
42 chromatography (HPLC), International Agency for Research on Cancer (IARC), Joint
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196
197
198
199
200
201
202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552
553
554
555
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
841
842
843
844
845
846
847
848
849
850
851
852
853
854
855
856
857
858
859
860
861
862
863
864
865
866
867
868
869
870
871
872
873
874
875
876
877
878
879
880
881
882
883
884
885
886
887
888
889
890
891
892
893
894
895
896
897
898
899
900
901
902
903
904
905
906
907
908
909
910
911
912
913
914
915
916
917
918
919
920
921
922
923
924
925
926
927
928
929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944
945
946
947
948
949
950
951
952
953
954
955
956
957
958
959
960
961
962
963
964
965
966
967
968
969
970
971
972
973
974
975
976
977
978
979
980
981
982
983
984
985
986
987
988
989
990
991
992
993
994
995
996
997
998
999
1000

Abstract

Fumonisin occurs mainly in maize, and produces alterations in sphingolipid metabolism, unbalancing the sphinganine (Sa) / sphingosine (So) ratio. This alteration has been proposed as a biomarker of fumonisin exposure. The objective of this study was to establish the urinary and plasmatic levels of Sa, So as well as, the ratio Sa/So from a sample of Catalonian population exposed to fumonisins at low levels. Firstly, plasma and urinary Sa and So levels and the ratio Sa/So were compared between two population groups, and after, urinary Sa and So levels from corn-food consumers and a control group were monitored for two weeks under controlled intake of corn-foods. Sa and So levels were determined in urine and blood samples using validated methods using HPLC with fluorescence detection. Significant differences were not found in urine samples when Sa/So ratios were compared from corn-food consumers and non-consumers, while significant differences were found in urine and plasma samples but evidence of the mechanism of action of fumonisins was not apparent. Through a time-course study, we have narrowed down the day in which the maximum alteration of Sa/So ratio should be expected in humans. In this paper we have reported some useful information to improve the design of studies to validate the ratio Sa/So as a possible biomarker of fumonisin exposure.

Introduction

Fumonisin B₁ (FB₁) and B₂ (FB₂) are mycotoxins produced by *Fusarium verticillioides* and *F. proliferatum* that commonly contaminate maize (Nelson et al. 1992). Fumonisin occur mainly in maize and maize-based foods, thus populations with high maize consumption can be exposed to significant amounts of these mycotoxins via the ingestion of fumonisin contaminated maize (Marasas 1996; Shephard et al. 1996; Visconti et al. 1996).

Acute and chronic toxicity of fumonisin has been demonstrated in several animal species, including carcinogenicity and cardiovascular toxic effects (Gelderblom et al. 1988, 1991; Howard et al. 2001; Shephard et al. 2007). FB₁ is a potent cancer promoter in rats after initiation with diethylnitrosamine and aflatoxin B₁ (Gelderblom et al. 1996). Human exposure to fumonisin contaminated commodities has been correlated with high rates of esophageal and liver cancer in South Africa and China (Sydenham et al. 1990; Yoshizawa et al. 1994) and more recently, with neuronal tube defects on the Texas-Mexico border with other possible risk factors (Hendricks et al. 1999).

Based on toxicological evidence, the International Agency for Research on Cancer (IARC) classified FB₁ as a possible human carcinogen (group 2B) (IARC 2002). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) evaluated fumonisins and allocated a provisional maximum tolerable daily intake (PMTDI) of 2 µg/kg body

1
2
3 weight/day of fumonisins. This value was determined on the basis of the overall non
4
5 observed effect level (NOEL) of 0.2 mg/kg body weight/day for renal toxicity in rats, and
6
7 the safety factor was 100 (JECFA 2001).
8
9

10
11
12 Natural occurrence of fumonisins in maize or maize-based foods has been widely studied
13
14 worldwide (Castelo et al., 1998) and several surveys have been conducted in maize-food
15
16 for human consumption marketed in Spain (Sanchis et al. 1994, 1995; Torres et al. 1998;
17
18 Castellá et al. 1999; Velluti et al. 2001; UdL-ACSA 2009). In the latest study conducted
19
20 in Catalonia to assess the incidence of mycotoxins in food for human consumption, 928
21
22 samples were purchased from the Catalonian market (in 2008 and 2009) and pooled in
23
24 370 composite samples to be analyzed. The commodities analyzed were beer, sweet-corn,
25
26 corn snacks, corn flakes, free-gluten pasta and bread and ethnic food. The authors
27
28 reported that the highest occurrence of fumonisins was found in beer (90 % of positive
29
30 samples), however those levels were low, while the incidence of these mycotoxins in
31
32 other cereal-based foods was moderated. The mean values of positive samples of corn
33
34 snacks and corn flakes were 119.1 ± 83.1 and 78.9 ± 27.9 $\mu\text{g}/\text{kg}$, respectively (UdL-ACSA
35
36 2009).
37
38
39
40
41
42
43
44
45

46 Fumonisin have a remarkable structural similarity to sphingolipids (Merrill and Sweeley
47
48 1996; Riley et al. 2001). This group of mycotoxins, especially FB₁, potently inhibits the
49
50 enzyme ceramide (CER) synthase which catalyzes the acylation of sphinganine (Sa) and
51
52 reacylation of sphingosine (So). The inhibition of CER synthase increases the
53
54 intracellular Sa and other sphingoid bases, highly cytotoxic compounds. This imbalance
55
56
57
58
59
60

1
2
3 has been proposed as the main responsible for the toxicity, and possibly carcinogenicity,
4 of FBs, based on mechanistic studies with cells cultures, and borne out by animal studies
5
6 (Wang et al. 1991; Norred et al. 1992; Merrill et al. 1993, 2001; Yoo et al. 1996; Riley et
7
8 al. 2001; Voss et al. 2006; Zitomer et al. 2009). Based on this biological perturbation,
9
10 elevation of Sa to So in tissues, urine and blood have been proposed as potential
11
12 biomarkers of fumonisin exposure in various animal species (Wang et al. 1992; Riley et
13
14 al. 1993; Morgan et al. 1997; Wang et al. 1999; van der Westhuizen et al. 2001; Kim et
15
16 al. 2006; Tran et al. 2006; Cai et al. 2007). Several studies have been conducted to assess
17
18 the effectiveness of this biomarker in humans, but results did not allow an accurate
19
20 validation (Hendricks et al. 1999; van der Westhuizen et al. 1999, 2008, 2010; Abnet et
21
22 al. 2001; Qiu and Liu 2001; Solfrizzo et al. 2004; Nikiema et al. 2008; Silva et al. 2009;
23
24 Xu et al. 2010). The individual Sa and So basal levels, as well as, the basal Sa/So ratio
25
26 vary depending on unknown parameters, being related with nutrition factors (Abnet et al.
27
28 2001; Shephard et al. 2007). The sensitivity of the correlation between fumonisin intake
29
30 and Sa/So has been demonstrated to be poor at low and very low doses in animals (< 1
31
32 mg/kg bw/day). Considering that the PMTDI is 2 µg/kg bw/day, low sensitivity should be
33
34 expected when we apply this biomarker in human population (Kim et al. 2006; Voss et al.
35
36 2006; Cai et al. 2007).

37
38
39
40
41
42
43
44
45
46
47
48 The objective of the work reported here was to study the urinary and plasma levels of Sa,
49
50 So as well as the ratio Sa/So from a sample of Catalonian population, exposed at low
51
52 levels of fumonisins, as a means to assess this ratio as a possible biomarker of fumonisin
53
54 intake in the region. This work is structured in two experimental sections: in the first,
55
56
57
58
59
60

1
2
3 plasma and urinary Sa and So levels and the ratio Sa/So were compared between two
4
5 population groups, in the second, urinary Sa and So levels from maize-food consumers
6
7 and a control group were monitored during two weeks under controlled intake of maize-
8
9 foods.
10
11

12 13 14 15 **Materials and methods**

16 17 **Study design and sampling**

18
19 This research project did not involve any risks for the volunteer donors; neither harmful
20
21 modification of usual dietary habits nor administration was included in methodology for
22
23 the subjects. Each participant was informed about the study rules and a signed
24
25 authorization was requested individually.
26
27
28
29
30
31

32 *Part 1. Urinary and plasmatic Sa/So ratio point estimates.*

33
34 The first attempt to know the urinary and plasmatic Sa and So levels, and the ratio Sa/So
35
36 from Catalonian population was designed to compare Sa, So levels and ratio Sa/So
37
38 between high consumers of maize food and low/non consumers.
39
40

41 *Part 1.1. Study of plasmatic sphingoid bases levels*

42
43 Blood samples were collected from 136 healthy adult volunteers during 2008, from
44
45 Catalonian population following approval from University of Lleida Ethical Council and
46
47 informed consent. Blood was extracted and stored (less than 2 hours) in Vacutainers®
48
49 with anticoagulant (EDTA) followed by centrifugation at 1000 g for 10 min, and finally,
50
51 the plasma was stored at -20°C until analysis (same month). Maize-food intake was
52
53 requested with a Food Frequency Questionnaire (FFQ) in order to determine the
54
55
56
57
58
59
60

1
2
3 approximate individual fumonisin intake. Fumonisin intake was estimated through the
4 combination of the consumption data with contamination data provided by UdL-ACSA
5 (2009). Two population groups were made depending on their estimated fumonisin
6 intake: high exposed and low or none exposed.
7
8
9
10
11

12
13
14
15 *Part 1.2. Urinary sphingoid base levels*
16

17 First morning urine from 89 volunteers was collected in sterile containers, during 2009,
18 according to Declaration of Helsinki. Urine samples were transported under refrigeration
19 and they were stored at -20°C until analysis. In order to estimate the individual fumonisin
20 intake, maize-food intake was requested through a FFQ and a 3 days record (R3) during
21 the days prior to the sample collection day. Fumonisin intake was estimated through the
22 combination of the consumption data with contamination data provided by UdL-ACSA
23 (2009). The population was grouped in high consumers and low consumers depending on
24 their maize dietary estimates. Finally, 7 urine samples were provided by esophageal
25 cancer sufferers from University Hospital Arnau de Vilanova (Lleida). These samples
26 were analyzed to determine Sa and So levels, and compared with the healthy group.
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43

44 *Part 2. Study of the urinary Sa and So time-course.*
45

46 To know the changes of urinary sphingolipid levels over time, two groups of volunteers
47 were monitored during 16 days. One group was composed of maize-food consumers
48 (exposed group n=24), and the other, by non consumers (control group n=12). The
49 exposed group was restricted of maize-food consumption during 16 days, with the
50 exception of the seventh day after restriction, when the maize-food intake was completely
51
52
53
54
55
56
57
58
59
60

1
2
3 free. The food items consumed were: home-made Mexican “tortillas”, corn snacks,
4
5 maize-based cake, sweet corn and beer purchased from a Catalonian market. A
6
7 representative sample of each maize-food consumed during that day, was kept and they
8
9 were analyzed by duplicated to determine the FB levels. The control group was restricted
10
11 to maize-food during the entire study period (See Fig 1).
12
13

14
15
16
17 [Insert Figure 1 about here, if possible]

18
19 (Title: Fig. 1. Diagram of urine sampling design and restriction periods performed in the
20
21 study to assess urinary Sa and So time-course.
22
23

24
25 Footnote: US: urine sample, R24: 24 hours record, FFQ: Food Frequency Questionnaire).
26
27

28
29 At the beginning of the experiment, dietary habits of the individuals were requested via a
30
31 FFQ. Maize-food intake during the free consumption day (day 0) by the exposed group
32
33 was requested with a 24 hour dietary using household sizes previously standardized. First
34
35 morning urine was sampled according to Declaration of Helsinki, from control group at
36
37 days -7, 0, 4 and 8, while in the exposed group, two sampling days (1 and 6) were added
38
39 in order to increase the accuracy. Urine samples were transported under refrigeration, and
40
41 they were stored at -20°C until analysis (during the same month). Sa and So levels were
42
43 determined for each urine samples.
44
45
46
47

48
49
50 Fumonisin analysis in food

51
52 Fumoniprep[®] immunoaffinity cleanup columns (IAC) (R-Biopharm, Rhône LTD
53
54 Glasgow, UK) were used to extract FB₁ and FB₂ from beer samples. A volume of 5 mL
55
56
57
58
59
60

1
2
3 of beer previously degassed in ultrasonic bath during 40 minutes was mixed with 15 mL
4 of phosphate buffer solution (PBS; 0.8 % NaCl, 0.12% Na₂HPO₄, 0.02% KH₂PO₄, 0.02%
5 KCl) and drained through the IAC. The column was washed with 20 mL of PBS solution
6 and fumonisins were eluted with 1.5 mL of methanol grade HPLC and 1.5 mL of milli-Q
7 water. Regarding solid maize-based samples, 10 g of ground sample was mixed with 1 g
8 NaCl, and 50 mL of extract solution (50% water, 25% methanol, 25% acetonitrile) for 20
9 minutes and filtered. 10 mL of filtered solution was diluted with 40 mL of PBS and
10 drained through the IAC and follows as described previously.
11
12

13
14
15
16
17
18
19
20
21
22 Fluorescent derivatives of FB₁ and FB₂ were obtained using pre-column derivatization
23 with an *o*-phthaldialdehyde (OPA) solution prepared diluting 40 mg of *o*-
24 phthaldialdehyde with 1 mL of methanol HPLC grade and mixed with 5 mL of
25 Na₂B₄O₇·10H₂O (0.1M) and 50 µL de 2-mercaptoethanol. Derivatization was conducted
26 mixing 200 µL of eluate with 200 µL of OPA solution for 30 seconds in vortex.
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Chromatography equipment: Separations Module Alliance 2695 Waters®, analytical
column Waters Spherisorb® 5µm ODS2, 4.6 x 150 mm, Multi λ Fluorescence Detector
Waters 2475®, kept at 35 °C and a flow-rate maintained at 1 mL/min. Mobile phase was
based on a methanol and 0.1M sodium dihydrogen phosphate (77:23, v/v) solution.
Excitation and emission wavelength was 335 nm and 440 nm, respectively.

Sphinganine and Sphingosine analysis in plasma

Plasma (500 µL) was deproteinized with methanol (2 mL) and the protein precipitate was
centrifuged down at 1200 g for 10 min at 10 °C. An aliquot of the sobrenatant (1.5 mL)
was mixed with 1.5 mL potassium chloride solution (0.8 %) and 50 µL potassium

1
2
3 hydroxide (1 M). The mixture was extracted with 4 mL of ethyl acetate by gentle rotation
4
5 in a blender for 20 min and the phases were separated by centrifugation at 1100 g for 15
6
7 min, as described by Castegnaro et al. (1998). The organic phase was evaporated to
8
9 complete dryness at 55°C under nitrogen. Dried samples were redissolved by vortex
10
11 shaking in 275 µL methanol-water solution (88:12) and derivatized for 35 min by
12
13 addition of 25 µL of OPA mixture. The derivatization mixture consisted of 50 mg OPA
14
15 dissolved in 1 mL of ethanol and mixed with 50 µL of mercaptoethanol and 48.95 mL of
16
17 boric acid solution (3 %) adjusted to pH 10.5 with potassium hydroxide (1 M) to obtain a
18
19 final volume of 50 mL. The derivatives were analyzed by HPLC with fluorescence
20
21 detection (excitation wavelength of 340 nm, emission wavelength of 455 nm), using a
22
23 Waters Spherisorb® 3 µm ODS2 4.5x250 mm column, kept at 35 °C and a flow-rate
24
25 maintained at 1 mL/min of methanol-water (88:12, v/v).
26
27
28
29
30
31
32
33

34 Sphinganine and sphingosine analysis in urine

35
36
37 Urinary samples were stored at -20°C in the dark before the analysis. Extraction of Sa and
38
39 So was performed using a method adapted from Castegnaro et al. (1996). To sum up, 20
40
41 mL of urine thawed sample were centrifuged at 2000 g for 15 minutes at 10 °C, in order
42
43 to isolate exfoliated cells. Cell pellets were re-suspended in 2 ml distilled water with 50
44
45 µl of potassium hydroxide (1 M). Following, 2 ml of ethyl acetate were added and mixed
46
47 vigorously using the vortex for 1 minute. Then, the mixture was centrifuged at 2000 g for
48
49 15 minutes, and the upper solvent layer was kept, while the aqueous phase was extracted
50
51 again. Finally, the mixed solvent layers containing sphingolipids were dried under a
52
53
54
55
56
57
58
59
60

1
2
3 nitrogen stream. Dried samples were analyzed as described for plasma samples, under the
4
5 same chromatographic conditions.
6
7
8
9

10 Validation of the analytical methods

11
12 The analytical methods used for fumonisins, Sa and So were assessed for selectivity,
13
14 linearity, and precision. Selectivity was checked by injecting 50 µl of mycotoxin standard
15
16 solutions three times before injecting extracted samples and comparing the peak retention
17
18 times and the fluorescence spectra of the substances that produced these peaks. Standard
19
20 curves were generated by linear regression of peak areas against concentrations.
21
22
23

24 Accuracy and recovery were established by determination of FB₁ and FB₂ levels, spiked
25
26 in samples of corn snacks, beer, and sweet corn; in the case of Sa and So, they were
27
28 spiked in urine and blood samples. Recovery was determined by comparing the absolute
29
30 responses of fumonisins, Sa and So, with the absolute responses of calibration standards.
31
32
33

34 The limit of detection (LOD) was considered as the mycotoxin and the sphingolipid
35
36 concentration that provides a signal equal to $b+3S_b$, where b is the intercept of the
37
38 calibration curve and S_b is the standard error of the estimate assuming to be the blank,
39
40 and the limit of quantification (LOQ) was considered equal to $3\times\text{LOD}$.
41
42
43

44 Recovery data, repeatability, limit of detection (LOD) and limit of quantification (LOQ)
45
46 of FB₁ and FB₂ in sweet corn, corn snacks and beer are shown in Table 1. These values
47
48 are in accordance to performance criteria established by Commission Regulation (EC) N°
49
50 401/2006 (European Commission 2006a).
51
52
53

54
55 [Insert Table 1 about here, if possible]
56
57
58
59
60

1
2
3
4
5
6 Method performance characteristics for Sa and So in blood are shown in Table 2. This
7
8 method showed recovery rates of So ranging from 92.2 ± 19.7 to 104.0 ± 12.8 %, while the
9
10 recovery rates for Sa were between 93.1 ± 13.4 and 98.3 ± 11.2 %.

11
12
13
14
15 [Insert Table 2 about here, if possible]
16
17
18
19

20 The method to determine the sphingoid bases in urinary samples was optimized in order
21
22 to obtain a low detection limit, due to the low concentration of sphingolipids expected to
23
24 be found in this matrix. Recovery rates, RSDr, LOQ and LOD are shown in Table 3.

25
26
27
28
29 [Insert Table 3 about here, if possible]
30
31
32
33

34 Statistical Analysis

35
36 Sa/So ratios were calculated individually by division of Sa and So levels from each
37
38 volunteer, and expressed as medians, means and standard deviations of ratios for each
39
40 group.
41
42

43 Mann-Whitney *U* test was used for two-group comparison and Kruskal-Wallis test was
44
45 used to compare more samples. Principal Component Analysis was conducted to obtain
46
47 matrix correlation from Sa/So ratio data and associated factors. Software SAS Enterprise
48
49 guide v2.0.0.417[®] and SAS v9.0.[®] were used in statistical analysis.
50
51
52
53
54

55 Results

56
57
58
59
60

1
2
3 Study of sphingoid base levels and ratios in plasma
4

5
6 In this first study, 136 blood donors were grouped in high maize-based food consumers
7
8 (68) and non consumers (68). The mean exposure to fumonisin estimated for the first
9
10 group was 0.23 ± 0.11 $\mu\text{g}/\text{kg}$ bw/day. Medians were 0.53 and 0.46 for maize-based food
11
12 consumers and non consumers, respectively. Although significant differences were
13
14 observed when the Sa/So ratios were compared, non statistically significant differences
15
16 were found between sphinganine levels ($P > 0.05$), the sphingosine decrease being the
17
18 most probable responsible of ratio variation in the exposed group (See Table 4).
19
20

21
22 Study of the sphingoid base levels and ratios in urine
23

24
25 In this cross-sectional study, 78 volunteers were selected to assess the urinary Sa and So
26
27 levels. Each volunteer was asked about dietary habits, through a FFQ and a R3.
28
29 Considering the estimated fumonisin intake, the population was divided in high and low
30
31 consumers in order to compare Sa and So levels and their ratios. The mean fumonisin
32
33 intake estimated through the R3, was 0.013 and 0.046 $\mu\text{g}/\text{kg}$ bw/day for males and
34
35 females, respectively, while these respective estimates were 0.089 and 0.057 $\mu\text{g}/\text{kg}$
36
37 bw/day when the estimation was made using the FFQ The most important bias sources
38
39 were that males overestimated significantly the beer consumption in comparison with the
40
41 R3 and the females underestimated the usual corn snacks consumption with this method.
42
43
44 Levels of Sa, So and Sa/So ratio and fumonisin intake estimated through the R3 are
45
46 shown in the Table 4.
47
48
49
50

51
52
53 [Insert Table 4 about here, if possible]
54
55
56
57
58
59
60

1
2
3 Significant differences were found between median ratios Sa/So from high and low
4 consumers ($P<0.05$), but no differences were found in Sa and So levels.
5
6

7
8 The urinary samples from esophageal cancer sufferers showed mean levels of Sa and So
9 to be 0.376 ± 0.471 and 0.208 ± 0.484 ng/mL, respectively, while the mean Sa/So ratio was
10
11
12 0.363 ± 0.458 , no significantly different from that of healthy population.
13
14

15 16 17 18 Urinary Sa/So ratio time-course

19
20 Fumonisin dietary intake of individuals was assessed combining food consumption data
21 with fumonisin levels on the maize-food consumed. Consumption data was recorded
22 during the day 0 (free consumption day), using previously standardized portions. Mean
23 levels of fumonisin contamination in corn snacks, Mexican “tortillas”, corn-based cake,
24 and sweet corn samples were 133.9, 99.3, 110.1 $\mu\text{g}/\text{kg}$ and non detectable level,
25 respectively. These values were far from EU limits of 400 $\mu\text{g}/\text{kg}$ (European Commission
26 2006b). Volunteers were classified in three groups, depending on total fumonisin intake
27 estimated during the “free maize-food consumption day”: high consumers, H, (>0.6
28 $\mu\text{g}/\text{kg}$ bw/day, mean 0.84 ± 0.26 $\mu\text{g}/\text{kg}$ bw/day); low consumers, L, (<0.6 $\mu\text{g}/\text{kg}$ bw/day,
29 mean 0.43 ± 0.12 $\mu\text{g}/\text{kg}$ bw/day) and non consumers, C, (control group, $n=12$). The high
30 consumers did not exceed the tolerable daily intake of 2 $\mu\text{g}/\text{kg}$ bw/day. The volunteer
31 population was 18 males and 18 females. 55 % of volunteers presented a body mass
32 index between 18.5 and 24.9 kg/m^2 (normal) and 45 % were overweight. Tobacco was
33 consumed by 32 % of the individuals.
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52

53
54 Mean levels of Sa and So, and mean ratios in urine samples from volunteer donors
55 collected during the restriction period, are shown in Table 5. The two volunteers excluded
56
57
58
59
60

1
2
3 from the study showed So and Sa basal levels markedly higher than the rest of the group
4
5 (40 fold greater than the mean group level).
6
7
8
9

10 [Insert Table 5 about here, if possible]
11
12
13

14
15 Median Sa levels at the beginning of the study were 0.27, 0.28 and 0.14 ng/mL, for non
16
17 consumers, low consumers and high consumers, respectively, and So median levels were
18
19 1.22, 0.68 and 0.33 ng/mL, respectively. The median Sa/So ratios were quite similar
20
21 between exposition groups, without statistically significant differences for these values
22
23 (0.25 to 0.70). During the first week of maize-food restriction, we did not observe
24
25 significant differences in the Sa/So ratios for any group, however, after the free maize-
26
27 food consumption day, statistically significant differences were observed in exposed
28
29 groups while no differences were observed in the control group with time. The maximum
30
31 increase of the Sa/So ratio was observed the fourth day after the free consumption day,
32
33 with mean values of 1.96 ± 2.24 and 2.52 ± 2.00 ng/mL for low and high consumers,
34
35 respectively, while the mean ratio for the control group was 0.67 ± 0.49 ng/mL (see figure
36
37 2). After the fourth day, the stabilization of the ratios was observed for these groups,
38
39 recovering initial values, without statistical differences among all groups (See Table 5).
40
41 This fact was confirmed by means of the correlation matrix of the ratios against the
42
43 estimated daily intake during the free consumption day. Principal component analysis of
44
45 Sa, Sa, Sa/So and fumonisin intake showed that the higher correlation should be expected
46
47 between estimated fumonisin intake and Sa/So ratio from day 4 ($r=0.3322$; $P<0.01$) with
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 low correlation with the other days. The mean Sa/So ratios through the time are
4
5 represented in the figure 2.
6
7

8
9
10 [Insert Figure 2 about here, if possible]

11
12 Title: Fig. 2. Time-course of mean Sa/So ratio for high exposed (H, \blacklozenge), low exposed
13
14 (L, \blacksquare) and non exposed (C, \blacktriangle).
15
16
17

18
19
20 Sa levels decreased during the first restriction week in each group, but increased after the
21
22 free consumption day in exposed groups (high and low consumers), while the level
23
24 slightly decreased in the control group. So levels decreased during the first week, after
25
26 day 0 the median values decreased slightly but no significant differences were found (See
27
28 Table 5).
29
30

31
32 The absolute modification of the sphingoid bases, as well as of the ratio, after the free
33
34 consumption day was quantified as the absolute difference (maximum – minimum)
35
36 among day 0 and day 8 (Table 6). Sa and So levels were slightly modified during this
37
38 period, without differences among exposure groups, neither significant differences were
39
40 observed in the increase of the Sa/So ratio.
41
42
43
44
45

46 [Insert Table 6 about here, if possible]
47
48
49

50 51 **4. Discussion**

52
53 Based on the mechanism of action, it has been observed that fumonisins inhibit CER
54
55 synthase, a disruption that leads to an increase of Sa levels and Sa/So ratio (Riley et al.
56
57
58
59
60

1
2
3 2001). It is due to the rapid elimination and low bioavailability of fumonisins, that it is
4 necessary to find an indirect indicator of human exposure to these toxins. Sa/So and Sa 1-
5 phosphate / So 1-phosphate ratios in tissues, urine and blood, have been proposed as
6 potential biomarkers in various animals (Wang et al. 1992, 1999; Morgan et al. 1997; van
7 der Westhuizen et al. 2001; Tran et al. 2006), these ratios being validated in F344 rats by
8 Cai et al. (2007), obtaining more sensitive results in urine than in serum for acute and
9 sub-chronic exposure to FB₁. However, no successful results have been found when this
10 biomarker has been assessed in human population, due to the low sensitivity when it is
11 applied over individuals (van der Westhuizen et al. 1999, 2008, 2010; Abnet et al. 2001;
12 Qiu and Liu 2001; Solfrizzo et al. 2004; Nikiema et al. 2008; Silva et al. 2009; Xu et al.
13 2010).

14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29 In Catalonia, **maize**-based food is not highly consumed; therefore the exposure of the
30 population to fumonisins is expected to be low, as reported in the Technical Report from
31 UdL-ACSA (2009). In the present study, we have estimated that fumonisin intake of the
32 volunteers from this region was in all cases below the PMTDI of 2 µg/kg bw/day,
33 including the high consumers, who showed maximum estimates of 1.04 and 1.42 µg/kg
34 bw/day. Other previous studies were conducted in regions where **maize** is highly
35 consumed, and estimated fumonisin intake has been estimated to be quite high; for
36 example, in some regions of South Africa the mean fumonisin intake was estimated to be
37 between 5.8 and 3.8 µg/kg bw/day (van der Westhuizen et al. 1999, 2008, 2010), and the
38 93 % of 43 volunteers from Huian (China) had their daily FB intakes above the PMTDI
39 of 2 µg/kg bw/day (Xu et al. 2010).
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 Concerning our cross-sectional studies, the mean plasmatic Sa and So levels were higher
4 than urinary levels, as reported previously (van der Westhuizen et al. 2008), while mean
5 ratios were slightly higher in urinary samples.
6
7
8
9

10 In both cross-sectional studies we have found significant differences between
11 ratios from exposed and non exposed groups, however no differences were found in
12 sphinganine levels. In the study performed with plasma, the main responsible of ratio
13 increase was elucidated through a decrease of So levels, with significant differences,
14 therefore no evidences of mechanism of action of fumonisins were found.
15
16
17
18
19
20
21

22 Esophageal cancer rates have been correlated with fumonisin exposure in China
23 and South Africa, to regions highly exposed to fumonisins (Chu and Li 1994; Zhang et
24 al., 1999; Wang et al., 2000; Rheeder et al., 1992). In northern Italy region, maize
25 consumption was correlated with higher rates of esophageal cancer than other regions
26 (Rossi et al., 1982; Franceschi et al., 1990), and presence of fumonisin-producing
27 *Fusarium* species in maize and polenta was lately reported (Logrieco et al., 1995; Pascale
28 et al., 1995). In this study seven urine samples from esophageal cancer sufferers were
29 analyzed and compared with healthy groups, and non differences were found in any case.
30
31 In our latest study, we have monitored the expected alteration of Sa and So levels in urine
32 from maize-food consumers after a free maize-food consumption day within a maize-
33 food restriction period. Significant differences were observed for the ratio Sa/So after the
34 free consumption day (day 0) for both exposed groups, while no differences were
35 observed in the control group. The maximum values of the ratios were observed at day 4
36 after the free consumption day. Previous studies conducted with animal species dosed
37 with fumonisins showed variable results concerning the day of maximum Sa/So ratio. For
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 example, the maximum peak of Sa/So in weaned piglets dosed with 5 mg/kg bw/day
4
5 showed the peak at 12 h (Dilkin et al. 2010), in rats dosed with 10 mg/kg bw/day the
6
7 maximum was observed at day 3 and day 5 (Garren et al. 2001; Cai et al. 2007), while in
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

example, the maximum peak of Sa/So in weaned piglets dosed with 5 mg/kg bw/day showed the peak at 12 h (Dilkin et al. 2010), in rats dosed with 10 mg/kg bw/day the maximum was observed at day 3 and day 5 (Garren et al. 2001; Cai et al. 2007), while in vervet monkeys dosed with 1 mg/kg bw/day the maximum was found to be the day 3 (Van der Westhuizen et al. 2001). The time period between fumonisin intake and maximum peak of the ratio Sa/So is an important data to validate a human biomarker that will permit a better design of sampling and dietary exposure assessment.

To date, the cross-sectional studies have shown poor correlation between fumonisin dietary intakes and Sa/So ratio in humans, however, successful results have been found in several animal studies. Thus, there are several drawbacks which prevent this biomarker to be applied to humans for epidemiologic purposes:

- 1) The individual Sa and So basal levels, as well as, the basal Sa/So ratio vary depending on unknown parameters, being related with nutrition factors (Abnet et al. 2001; Shephard et al. 2007). Therefore, the absolute ratio could not be a good predictor of fumonisin intake.
- 2) The sensitivity of the correlation between fumonisin intake and Sa/So has been demonstrated to be poor at low and very low doses in animals (< 1 mg/kg bw/day). Considering that the PMTDI is 2 µg/kg bw/day, low sensitivity should be expected when we apply this biomarker in human population. The Sa-P and So-P have been proposed to monitor the exposure of fumonisins, being more sensitive than the original sphingoid bases, therefore, could be suitable to use in human epidemiological studies for low-level exposed population (Kim et al. 2006; Voss et al. 2006; Cai et al. 2007).

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
- 3) To reach a realistic correlation between sphingoid base levels and fumonisin intake in human populations, it is required to use reliable analytical and consumption data (Willet 1998). Improved analytical methods to determine Sa and So are reliable in urine and blood and likewise the methods to determine the FB levels in food. However, the dietary intake assessment methods used in previous studies do not report on their accuracy or reliability.
- 4) Finally, the most commonly used method to assess dietary intake has been the food frequency questionnaire (FFQ); it is the most comfortable method for researchers and volunteers. If we consider that the maximum Sa/So ratio has been closely correlated with a specific consumption day in animals (dose day) (Garren et al. 2001; van der Westhuizen et al. 2001; Dilkin et al. 2010), and reversible after that point, the dietary intake methods should assess rigorously those foods consumed 4-5 days before the urine sampling. Therefore, the dietary record could be a more accurate method to assess the fumonisin intake than de FFQ.

39 **Conclusions**

40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

To our knowledge, this is the first study conducted in Spain to assess the sphingoid base levels and ratios in plasma and urine from a maize-food consumer population. We have proved that the volunteers were not exposed to high levels of fumonisins, in all cases below PMTDI of 2 $\mu\text{g}/\text{kg}$ bw/day (maximum value of 1.4 $\mu\text{g}/\text{kg}$ bw/day). The analytical method to determine Sa and So in urine and plasma was reliable, showing good recovery and reproducibility. The results showed higher Sa and So levels in plasma than in urine, and significant differences were shown when males were compared to females.

1
2
3 Concerning Sa/So ratios from maize-food consumers and non-consumers, significant
4 differences were found in urine and plasma samples but evidences of mechanism of
5 action of fumonisins were not apparent. Through time-course study, we have narrowed
6 down the day in which the maximum alteration of Sa/So ratio should be expected in
7 humans.
8
9

10
11
12
13
14
15
16
17 In this paper we have reported some useful information to improve the design of studies
18 to validate the ratio Sa/So as a possible biomarker of fumonisin exposure. However, more
19 studies are required to better understand the use of this biomarker with human population,
20 mainly, to improve the accuracy at low levels of exposure.
21
22
23
24
25
26
27

28 29 **Acknowledgements**

30
31 The authors would like to acknowledge Exposure Assessment of Spanish Population to
32 *Fusarium* Toxins Project, National Plan of Spanish Government (AGL2008-05030-C02-
33 01), Catalanian Food Safety Agency of 'Generalitat de Catalunya' Health Department
34 and University of Lleida for their financial support.
35
36
37
38
39
40
41
42

43 **References**

44
45 Abnet CC, Borkowf CB, Qiao Y-L, Albert PS, Wang E, Merrill AH, Mark SD, Dong
46 ZW, Taylor PR, Dawsey SM. 2001. A cross-sectional study of human serum
47 sphingolipids, diet and physiologic parameters. J Nutr. 131:2748-2752.
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 Cai Q, Tang L, Wang J-S. 2007. Validation of fumonisin biomarkers in F344 rats.
4
5 Toxicol Appl Pharmacol. 225:28-39.
6
7

8
9
10 Castegnaro M, Garren L, Gaucher I, Wild CP. 1996. Development of a new method for
11
12 the analysis of sphinganine and sphingosine in urine and tissues. Nat Tox. 4:284-290.
13
14

15
16
17 Castegnaro M, Garren L, Galendo D, Gelderblom WCA, Chelule P, Dutton MF, Wild
18
19 CP. 1998. Analytical method for the determination of sphinganine and sphingosine in
20
21 serum as a potential biomarker for fumonisin exposure. J Chromatogr B. 720:15-24.
22
23
24

25
26
27 Castellá G, Bragulat MR, Cabañes FJ. 1999. Surveillance of fumonisins in maize-based
28
29 feed and cereals from Spain. J Agr Food Chem. 47:707-710.
30
31
32

33
34
35 Castelo MM, Summer SS, Bullerman LB. 1998. Occurrence of fumonisins in corn-based
36
37 food products. J Food Prot. 61:704-707.
38
39

40
41
42 Chu FS, Li GY. 1994. Simultaneous occurrence of fumonisin B1 and other mycotoxins in
43
44 moldy corn collected in the People's Republic of China in regions with high incidences
45
46 of esophageal cancer. Appl Environ Microbiol. 60:847-852.
47
48

49
50
51 Dilkin P, Direito G, Simas MM, Mallmann CA, Corrêa B. 2010. Toxicokinetics and
52
53 toxicological effects of single oral dose of fumonisin B1 containing *Fusarium*
54
55 *verticillioides* culture material in weaned piglets. Chem Biol Interact. 185:157-62.
56
57
58
59
60

1
2
3
4
5
6 European Commission. 2006a. Commission Regulation (EC) N° 401/2006 of 23 February
7
8 2006. OJEU. L70:12-34.
9

10
11
12 European Commission. 2006b. Commission Regulation (EC) N° 1881/2006 of 19
13
14 December 2006. OJEU. L364:5-24.
15
16

17
18
19 Franceschi S, Bidoli E, Barón AE, La Vecchia C. 1990. Maize and risk of cancer of the
20
21 oral cavity, pharynx and esophagus in northeastern Italy. J Natl Cancer Inst. 82:1407–
22
23 1411.
24
25

26
27
28
29 Garren L, Galendo D, Wild CP, Castegnaro M. 2001. The induction and persistence of
30
31 altered sphingolipid biosynthesis in rats treated with fumonisin B₁. Food Addit Contam.
32
33 18:850-6.
34
35

36
37
38 Gelderblom WCA, Jaskiewicz K, Marasas WFO, Thiel PG, Hora RM, Vleggar R, Kriek
39
40 NPJ. 1988. Fumonisin—Novel mycotoxins with cancer-promoting activity produced by
41
42 *Fusarium moniliforme*. Appl Environ Microbiol. 54:1806–1811.
43
44
45

46
47
48 Gelderblom WCA, Kriek NPJ, Marasas WFO, Thiel PG. 1991. Toxicity and
49
50 carcinogenicity of the *Fusarium moniliforme* metabolite, fumonisin B₁ in rats.
51
52 Carcinogenesis. 12:1247–1251.
53
54
55

1
2
3 Gelderblom WC, Snyman SD, Lebepe-Mazur S, van der Westhuizen L, Kriek NP,
4
5 Marasas WF. 1996. The cancer-promoting potential of fumonisin B₁ in rat liver using
6
7 diethylnitrosamine as a cancer initiator. *Cancer Lett.* 109:101-108.
8
9

10
11
12 Hendricks KA, Simpson JS, Larsen RD. 1999. Neural tube defects along the Texas-
13
14 Mexico border, 1993-1995. *Am J Epidemiol.* 149:1119-1127.
15
16

17
18
19 Howard PC, Eppley RM, Stack ME, Warbritton A, Voss KA, Lorentzen RJ, Kovach RM,
20
21 Bucci TJ. 2001. Fumonisin B₁ carcinogenicity in a two-year feeding study using F344
22
23 rats and B6C3F₁ mice. *Environ Health Perspect.* 109:277-282.
24
25
26

27
28
29 IARC (International Agency for Research on Cancer). 2002. IARC Monographs on the
30
31 Evaluation of Carcinogenic Risks to Humans, vol. 82. IARC, Lyon, France, p. 301-366.
32
33

34
35
36 Joint FAO/WHO Expert Committee on Food Additives (JECFA). 2001. Evaluation of
37
38 certain mycotoxins in food: fifty-sixth report of the Joint FAO/WHO Expert Committee
39
40 on Food Additives. Geneva, Switzerland.
41
42

43
44
45 Kim D-H, Yoo H-S, Lee Y-M, Kie J-H, Jang S, Oh S. 2006. Elevation of sphinganine 1-
46
47 phosphate as a predictive biomarker for fumonisin exposure and toxicity in mice. *J*
48
49 *Toxicol Env Heal A.* 69:2071-2082.
50
51
52

1
2
3 Logrieco A, Moretti A., Ritieni A, Bottalico A, Corda P. 1995. Occurrence and
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Logrieco A, Moretti A., Ritieni A, Bottalico A, Corda P. 1995. Occurrence and
toxigenicity of *Fusarium proliferatum* from preharvest maize ear rot, and associated
mycotoxin, in Italy. *Plant Dis.* 79:727–731.

Marasas WFO. 1996. Fumonisin: History, world-wide occurrence and impact. *Adv Exp
Med Biol.* 392:1-17.

Merrill AHJ, Wang E, Gilchrist DG, Riley RT. 1993. *Advances in Lipid Research:
Sphingolipids and Their Metabolites.* Orlando: Academic Press. Fumonisin and other
inhibitors of *de novo* sphingolipid biosynthesis; p. 215–234.

Merrill AH, Sweeley CC, 1996. *Biochemistry of Lipids, Lipoproteins and Membranes.*
New York: Elsevier. Sphingolipids: metabolism and cell signaling; p. 43–73.

Merrill JAH, Sullards MC, Wang E, Voss KA, Riley RT. 2001. Sphingolipid metabolism:
Roles in signal transduction and disruption by fumonisins. *Environ Health Perspect.*
109:283-289.

Morgan MK, Schroeder JJ, Rottinghaus GE, Powell DC, Bursian SJ, Aulerich RJ. 1997.
Dietary fumonisins disrupt sphingolipid metabolism in mink and increase the free
sphinganine to sphingosine ratio in urine but not in hair. *Vet Hum Toxicol.* 39:334–336.

1
2
3 Nelson PE, Plattner RD, Shackelford DD, Desjardins AE. 1992. Fumonisin B₁ production
4 by *Fusarium* species other than *F. moniliforme* in section *Liseola* and by some related
5 species. Appl Environ Microbiol. 58:984-989.
6
7
8
9

10
11
12 Nikiema PA, Worilow L, Traore AS, Wild CP, Turner PC. 2008. Fumonisin exposure
13 and the sphinganine/sphingosine ratio in urine, serum and buccal cells in adults from
14 Burkina Faso, West Africa. World Mycotox J. 1:483-491.
15
16
17
18
19

20
21
22 Norred WP, Plattner RD, Vesonder RF, Bacon CW, Voss KA. 1992. Effects of selected
23 secondary metabolites of *Fusarium moniliforme* on unscheduled synthesis of DNA by rat
24 primary hepatocytes. Food Chem Toxicol. 30:233-237.
25
26
27
28
29

30
31
32 Pascale M, Doko MB, Visconti A. 1995. Proceedings of the 2nd National Congress on
33 Food Chemistry. Messina: La Grafica Editoriale. Determination of fumonisins in polenta
34 by high performance liquid chromatography; p. 1067-1071.
35
36
37
38
39

40
41 Qiu M, Liu X. 2001. Determination of sphinganine, sphingosine and Sa/So ratio in urine
42 of humans exposed to dietary fumonisin B₁. Food Addit Contam. 18:263-269.
43
44
45
46
47

48
49 Rheeder JP, Marasas WFO, Thiel PG, Sydenham EW, Shephard GS, Van Schalkwyk DJ.
50 1992. *Fusarium moniliforme* and fumonisins in corn in relation to human esophageal
51 cancer in Transkei. Phytopathology 82:353-357.
52
53
54
55
56
57
58
59
60

1
2
3 Riley RT, An NH, Showker JL, Yoo HS, Norred WP, Chamberlain WJ, Wang E, Merrill
4 JAH, Motelin G, Beasley VR, et al. 1993. Alteration of tissue and serum sphinganine to
5 sphingosine ratio: an early biomarker of exposure to fumonisin-containing feeds in pigs.
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Toxicol Appl Pharmacol. 118:105–112.

Riley RT, Enongen E, Voss KA, Norred WP, Meredith FI, Sharma RP, Williams D,
Merrill AHJ. 2001. Sphingolipid perturbations as mechanisms for fumonisin
carcinogenesis. Environ Health Perspect. 109:301–308.

Rossi M, Ancona E, Mastrangelo G, Solimbergo D, Paruzzolo P, Assarini G, Sorrentina
P, Peracchia A. 1982. Epidemiologic findings in esophageal cancer in the Veneto region.
Minerva Med. 73:1531–1540.

Sanchis V, Abadias M, Oncins L, Sala N, Viñas I, Canela R. 1994. Occurrence of
fumonisins B₁ and B₂ in corn-based products from the Spanish market. Appl Environ
Microbiol. 60:2147–2148.

Sanchis V, Abadias M, Oncins L, Sala N, Viñas I, Canela R. 1995. Fumonisins B₁ and B₂
and toxigenic *Fusarium* strains in feeds from the Spanish market. Int J Food Microbiol.
27:37–44.

Shephard GS, Thiel PG, Storckenstrom S, Sydenham EW. 1996. Worldwide survey of
fumonisin contamination of corn and corn-based products. J AOAC Int. 79:671–687.

1
2
3
4
5
6 Shephard GS, Van der Westhuizen L, Sewram V. 2007. Biomarkers of exposure to
7
8 fumonisin mycotoxins: a review. *Food Addit Contam.* 24:1196-1201.
9

10
11
12 Silva LJG, Lino CM, Pena A. 2009. Sphinganine-sphingosine ratio in urine from two
13
14 Portuguese populations as biomarker to fumonisins exposure. *Toxicol* 54:390-398.
15
16

17
18
19 Solfrizzo M, Chulze SN, Mallmann C, Visconti A, De Girolamo A, Rojo F, Torres A.
20
21 2004. Comparison of urinary sphingolipids in human populations with high and low
22
23 maize consumption as a possible biomarker of fumonisin dietary exposure. *Food Addit*
24
25 *Contam.* 21:1090–1095.
26
27

28
29
30
31 Sydenham EW, Thiel PG, Marasas WFO, Shephard GS, Schalkwyk DJ, Koch KR. 1990.
32
33 Natural occurrence of some *Fusarium* mycotoxins in corn from low and high esophageal
34
35 cancer prevalence areas of Transkei, Southern Africa. *J Agric Food Chem.* 38:1900–
36
37 1903.
38
39

40
41
42
43 Torres MR, Sanchis V, Ramos AJ. 1998. Occurrence of fumonisins in Spanish beers
44
45 analysed by an enzyme-linked immunosorbent assay method. *Int J Food Microbiol.*
46
47 39:139-143.
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 Tran ST, Tardieu D, Auvergne A, Bailly JD, Babile R, Durand S, Benard G, Guerre P.
4
5 2006. Serum sphinganine and the sphinganine to sphingosine ratio as a biomarker of
6
7 dietary fumonisins during chronic exposure in ducks. *Chem Biol Interact.* 160:41–50.
8
9

10
11
12 University of Lleida – Catalanian Food Security Agency (UdL-ACSA). 2009. Project to
13
14 Assess the Exposure of Catalanian Population to the Mycotoxins. Internal Technical
15
16 Report June 2009. Lleida. Applied Microbiology Group/ Food Technology Department/
17
18 University of Lleida. Supported by Catalanian Food Security Agency.
19
20
21

22
23
24 Van der Westhuizen L, Brown NL, Marasas WF, Swanevelde, S, Shephard GS. 1999.
25
26 Sphinganine/sphingosine ratio in plasma and urine as a possible biomarker for fumonisin
27
28 exposure in humans in rural areas of Africa. *Food Chem Toxicol.* 37:1153–1158.
29
30
31

32
33
34 Van der Westhuizen L, Shephard GS, van Schalkwyk DJ. 2001. The effect of a single
35
36 gavage dose of fumonisin B₁ on the sphinganine and sphingosine levels in vervet
37
38 monkeys. *Toxicol* 39:273–281.
39
40
41

42
43
44 Van der Westhuizen L, Shephard GS, Rheeder JP, Somdyala NIM, Marasas WFO. 2008.
45
46 Sphingoid base levels in humans consuming fumonisin-contaminated maize in rural areas
47
48 of the former Transkei, South Africa: A cross-sectional study. *Food Addit Contam A*
49
50 25:1385-1391.
51
52
53
54
55
56
57
58
59
60

1
2
3 Van der Westhuizen L, Shephard GS, Rheeder JP, Burger H-M. 2010. Individual
4 fumonisin exposure and sphingoid base levels in rural populations consuming maize in
5 South Africa. *Food Chem Toxicol.* 48:1698-1703.
6
7
8
9

10
11
12 Velluti A, Marín S, Sanchis V, Ramos AJ. 2001. Note. Occurrence of fumonisin B₁ in
13 Spanish corn-based foods for animal and human consumption. *Food Sci Technol Int.*
14
15 7:433-437.
16
17
18
19

20
21
22 Visconti A, Doko MB, Solfrizzo M, Pascale M, Boenke A. 1996. European
23 Intercomparison Study for the Determination of Fumonisin in Maize. *Mikrochim Acta*
24
25 123:55-61.
26
27
28
29

30
31
32 Voss KA, Riley R, Dunn C, Christopher Corton J. 2006. The role of tumor necrosis factor
33 alpha and the peroxisome proliferator-activated receptor alpha in modulating the effects
34 of fumonisin in mouse liver. *Toxicology* 222:165-74.
35
36
37
38
39

40
41 Wang E, Norred WP, Bacon CW, Riley RT, Merrill AHJ. 1991. Inhibition of
42 sphingolipid biosynthesis by fumonisins: implications for diseases associated with
43
44 *Fusarium moniliforme*. *J Biol Chem.* 266:14486–14490.
45
46
47
48
49

50
51 Wang E, Ross PF, Wilson TM, Riley RT, Merrill JAH. 1992. Increases in serum
52 sphingosine and sphinganine and decreases in complex sphingolipids in ponies given feed
53
54
55
56
57
58
59
60

1
2
3 containing fumonisins, mycotoxins produced by *Fusarium moniliforme*. J Nutr.
4
5 122:1706–1716.
6
7

8
9
10 Wang E, Riley RT, Meredith FI, Merrill JAH. 1999. Fumonisin B₁ consumption by rats
11
12 causes reversible, dose-dependent increases in urinary sphinganine and sphingosine. J
13
14 Nutr. 129:214–220.
15
16

17
18
19 Wang H, Wei H, Ma J, Luo X. 2000. The fumonisin B₁ content in corn from North
20
21 China, a high-risk area of esophageal cancer. J Environ Pathol Toxicol Oncol. 19:139–
22
23 141.
24
25
26

27
28
29 Willett W. 1998. Nutritional Epidemiology. 2nd ed. New York: Oxford University Press.
30
31

32
33
34 Xu L, Cai Q, Tang L, Wang S, Hu X, Su J, Sun G, Wang J-S. 2010. Evaluation of
35
36 fumonisin biomarkers in a cross-sectional study with two high-risk populations in China.
37
38 Food Addit Contam A 27:1161-1169.
39
40

41
42
43 Yoo H-S, Norred WP, Showker JL, Riley RT. 1996. Elevated sphingoid bases and
44
45 complex sphingolipid depletion as contributing factors in fumonisin-induced cytotoxicity.
46
47 Toxicol Appl Pharmacol. 138:211–218.
48
49
50

1
2
3 Yoshizawa T, Yamashita A, Luo Y. 1994. Fumonisin occurrence in corn from high- and
4 low-risk areas for human esophageal cancer in China. *Appl Environ Microbiol.* 60:1626–
5
6 1629.
7
8
9

10
11
12 Zhang H, Nagashima H, Goto T. 1997. Natural occurrence of mycotoxins in corn samples
13 from high and low risk areas for human esophageal cancer in China. *Mycotoxins* 44:29–
14
15 35.
16
17
18
19

20
21
22 Zitomer NC, Mitchell T, Voss KA, Bondy GS, Pruett ST, Garnier-Amblard EC,
23
24 Liebeskind LS, Park H, Wang E, Sullards MC, Merrill AHJ, Riley RT. 2009. Ceramide
25
26 synthase inhibition by fumonisin B1 causes accumulation of 1-deoxysphinganine: a novel
27
28 category of bioactive 1-deoxysphingoid bases and 1-deoxydihydroceramides
29
30 biosynthesized by mammalian cell lines and animals. *J Biol Chem.* 284:4786-95.
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Table 1. Method performance characteristics for fumonisin B₁ and B₂

Food Matrix	n	FB ₁				FB ₂			
		LOD/LOQ	Spiked level	Recovery*	RSDr	LOD/LOQ	Spiked level	Recovery	RSDr
		µg/kg	µg/kg	%	%	µg/kg	µg/kg	%	%
Sweet Corn	3	5.2/15.6	266	101.5±11.8	11.60	5.2/15.6	133	104.0±7.3	7.04
Corn Snacks	3	5.2/15.6	266	76.5±11.5	15.01	5.2/15.6	133	109.7±12.3	11.30
Beer	5	3.9/11.7	200	93.0±14.0	15.00	3.9/11.7	100	108.0±10.0	10.00

* : Mean ± Standard Deviation

Table 2. Method performance characteristics for sphingosine and sphinganine in plasma

	LOQ/LOD ng/mL	Spiked level ng/mL	n	Recovery* %	RSDr %
Sphingosine	0.15/0.048	5	4	98.9±6.4	6.4
	0.15/0.048	20	4	92.2±19.7	21.3
	0.15/0.048	40	4	104.0±12.8	12.3
Sphinganine	0.14/0.047	2.5	4	96.8±9.7	9.3
	0.14/0.047	12	4	98.3±11.2	11.4
	0.14/0.047	24	4	93.1±13.4	14.3

*: Mean ± Standard Deviation

Table 3. Method performance characteristics for sphingosine and sphinganine in urine

	LOD ng/mL	Spiked level ng/mL	n	Recovery* %	RSDr %
Sphingosine	0.04	5	5	122.9±5.5	4.5
	0.04	40	5	126.9±18.2	14.4
Sphinganine	0.02	5	5	107.6±5.6	5.2
	0.02	40	5	104.3±9.3	8.9

*: Mean ± Standard Deviation

Table 4. Sphinganine (Sa) and sphingosine (So) levels in urine and plasma, and the Sa/So ratio in population from Catalonia (Spain), from the cross-sectional studies.

Group	n	Mean Fumonisin intake µg/kg bw/day	Sa* ng/mL	So* ng/mL	Ratio* Sa/So
<i>Levels in urine</i>					
Low and non consumers	43	0.02±0.02	0.38 (0.95±2.15) ^A	0.83 (2.57±5.02) ^A	0.40 (0.55±0.47) ^A
Consumers	35	0.14±0.83	0.26 (1.29±2.15) ^A	0.57 (2.59±0.85) ^A	0.56 (0.62±0.47) ^B
<i>Levels in plasma</i>					
Non consumers	68	0.00	4.12 (6.5±9.2) ^A	8.51 (14.3±16.5) ^A	0.46 (0.45±0.12) ^A
Consumers	68	0.23±0.11	3.14 (4.1±3.6) ^A	5.89 (7.8±6.8) ^B	0.53 (0.54±0.16) ^B

*Median (Mean ± Standard Deviation).

^(A) Capital letter: different letters mean significant differences between groups, when we compare non consumers with consumers ($P < 0.05$; Mann-Whitney U test)

Table 5. Time-course of median sphinganine and sphingosine levels, and Sa/So ratios (in ng/mL).

	Day -7	Day 0	Day 1	Day 4	Day 6	Day 8
<i>Ratio Sa/So</i>						
Control	0.25 ^{aA}	0.62 ^{aA}		0.52 ^{aA}		0.45 ^{aA}
Low exposed	0.70 ^{aAB}	0.26 ^{bA}	0.27 ^{aAB}	1.10 ^{abB}	0.43 ^{aA}	0.47 ^{aA}
High Exposed	0.51 ^{aA}	0.39 ^{abA}	0.80 ^{aA}	2.43 ^{bB}	0.98 ^{bAB}	0.45 ^{aAB}
<i>Sphingosine</i>						
Control	1.23 ^{aA}	0.53 ^{aA}		0.30 ^{aA}		0.21 ^{aA}
Low exposed	0.68 ^{aA}	0.45 ^{aA}	0.36 ^{aA}	0.19 ^{aA}	0.28 ^{aA}	0.32 ^{aA}
High Exposed	0.33 ^{aA}	0.20 ^{aA}	0.15 ^{aA}	0.08 ^{aA}	0.12 ^{aA}	0.31 ^{aA}
<i>Sphinganine</i>						
Control	0.27 ^{aA}	0.14 ^{aA}		0.12 ^{aA}		0.16 ^{aA}
Low Exposed	0.28 ^{aA}	0.13 ^{aA}	0.12 ^{aA}	0.24 ^{aA}	0.19 ^{aA}	0.15 ^{aA}
High Exposed	0.14 ^{aA}	0.10 ^{aA}	0.19 ^{aA}	0.19 ^{aA}	0.12 ^{aA}	0.16 ^{aA}

Control group (non consumers), Low exposed (< 0.6 µg/kg bw/day), High exposed (> 0.6 µg/kg bw/day).

^(A) Capital letter: in each row, different letters mean significant differences among days ($P < 0.05$, Kruskal-Wallis test)

^(a) Lower case letter: in each column, for each category, different letters mean significant differences between groups ($P < 0.05$, Kruskal-Wallis test)

Table 6. Absolute variation of sphinganine and sphingosine urinary levels from volunteers under restricted conditions, variation was accounted between day 0 and day 8 (in ng/mL).

Group	Sa			So			Sa/So		
	max	min	median (mean±sd)	max	min	median (mean±sd)	max	min	median (mean±sd)
Control	1.30	0.02	0.17 (0.27±0.35) ^a	2.31	0.01	0.28 (0.56±0.67) ^a	1.76	0.09	0.96 (0.90±0.55) ^a
Low Exposed	0.62	0.11	0.23 (0.28±0.18) ^a	1.77	0.10	0.81 (0.83±0.51) ^a	7.56	0.12	0.98 (2.09±2.65) ^a
High Exposed	0.80	0.10	0.15 (0.26±0.22) ^a	1.69	0.08	0.27 (0.45±0.48) ^a	6.56	0.44	1.06 (2.73±2.32) ^a

Control group (non consumers), Low exposed (< 0.6 µg/kg bw/day), High exposed (> 0.6 µg/kg bw/day).

^(a) Lower case letter: different letters mean significant differences when we compare categories ($P < 0.05$, Kruskal-Wallis test)

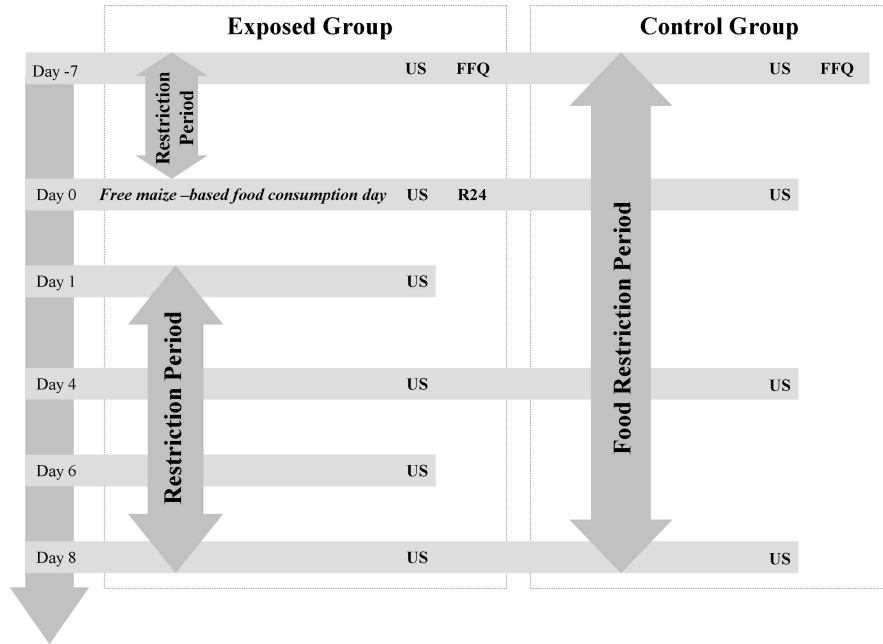


Figure 1.

Title: Diagram of urine sampling design and restriction periods performed in the study to assess urinary Sa and So time-course.

Footnote: US: urine sample, R24: 24 hours record, FFQ: Food Frequency Questionnaire

1418x1064mm (55 x 55 DPI)

Only

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

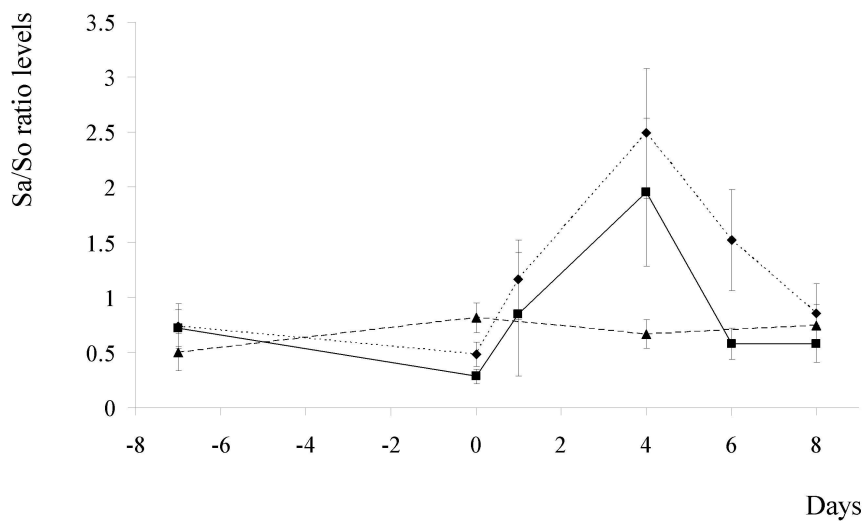


Figure 2
Title: Time-course of mean Sa/So ratio for high exposed (H,), low exposed (L,) and non exposed (C,).

1418x958mm (55 x 55 DPI)

1
2
3 **Figure Captions**
4
5

6
7 **Figure 1**

8
9 Title: Diagram of urine sampling design and restriction periods performed in the study to
10 assess urinary Sa and So time-course.
11
12

13 Footnote: US: urine sample, R24: 24 hours record, FFQ: Food Frequency Questionnaire
14
15

16
17
18 **Figure 2**
19

20 Title: Time-course of mean Sa/So ratio for high exposed (H, \blacklozenge), low exposed (L, \blacksquare)
21 and non exposed (C, \blacktriangle).
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60