New Biomarkers to Monitor the Dietary Consumption of Isothiocyanates

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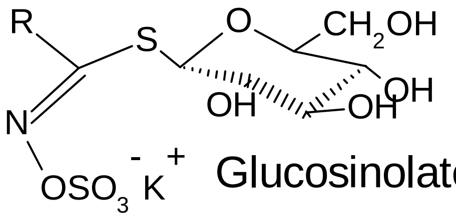
Introduction: Isothiocyanates in the diet?

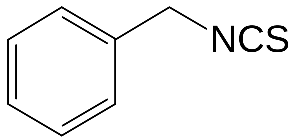
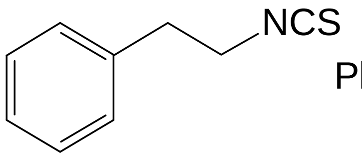
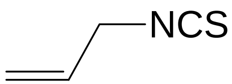
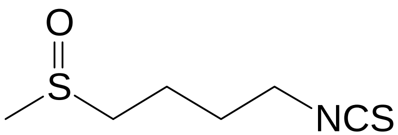


Glucosinolate (GL)

1. myrosinase
2. Lossen rearrangement

→ R-NCS + Glucose + $KHSO_4$

Isothiocyanate (ITC)

Glucosinolate (Precursor)		**Isothiocyanate**	**Food Sources**
Glucotropaeolin		Benzyl-Isothiocyanate (BITC)	Cabbage, garden cress, Indian cress
Gluconasturtiin		Phenethyl-Isothiocyanate (PEITC)	Watercress
Sinigrin		Allyl-Isothiocyanate (AITC)	Cabbage,horseradish, mustard
Glucoraphanin		Sulforaphane (SFN)	Broccoli sprouts, broccoli, Brussels sprouts, cabbage

INTRODUCTION

Are isothiocyanates (ITC) good for you?

- **ITCs found in cruciferous vegetables have demonstrated cancer preventive activity** in animals, and increased dietary intake of ITCs has been shown to be associated with a reduced cancer risk in humans.
- ITCs exert their cancer **chemopreventive action by modulating the activities of phase I and phase II drug metabolism enzymes**.
- ITCs and their thiol conjugates **inhibit the cell cycle and cause apoptotic cell death**, possibly by activation of vital signal transduction pathways.

- **ITC-protein adducts account for 87% of total cellular ITC-uptake** after 4h of treatment. The time course of this **protein binding correlated well with the inhibition of proliferation and the induction of apoptosis.** This suggests that cellular protein adducts of ITC may be an early event for apoptosis induction.

Clarke et al (2008). *Cancer Lett* **269**, 291-30 Slocum and Kensler (2011)*Arch Toxicol* **85**:273–2844**.**

Ho et al (2009). *J Nutr*, ***139, 2393-6.*** Conaway et al. (2002) *Curr. Drug Metab.*, **3**, 233-55.

Groopman et al (2008). *Annu Rev Public Health*, ***29, 187-203.*** Egner et al (2011) *Cancer Prev Res*, ***4, 384-95.***

OBJECTIVES

•Most **epidemiological studies on the relation of diet and cancer** have relied on the information collected with **questionnaires** to monitor the food intake. This is not very accurate.

• **Urinary metabolites might provide the exposure history of the last 24h**, if the urine of the full next day is collected (spot urine samples are not a reliable exposure marker). However, this is not feasible in large epidemiological studies. Furthermore the mercapturic acids of ITC in urine are not stable.

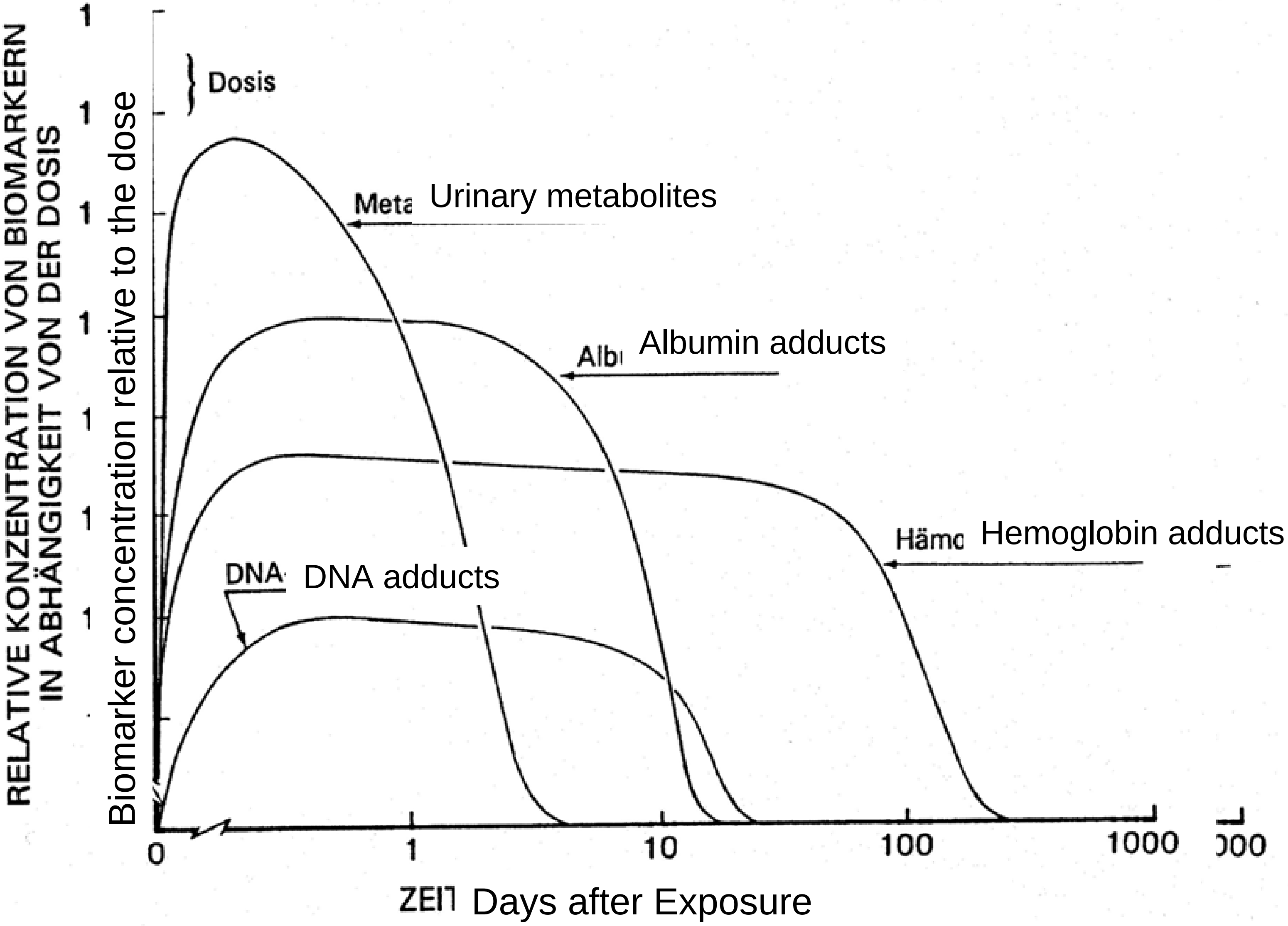
• **Stable biomarkers are needed which reflect a larger time span of the ITC-exposure history.** Hemoglobin adducts and albumin adducts have a lifetime of 120 days and a half-life of 20-25 days, respectively

• Thus, we propose to develop a **method to determine stable reaction products of ITCs with albumin and hemoglobin** in human.

•The method will be **tested on human subjects after controlled cruciferous vegetables intake.**

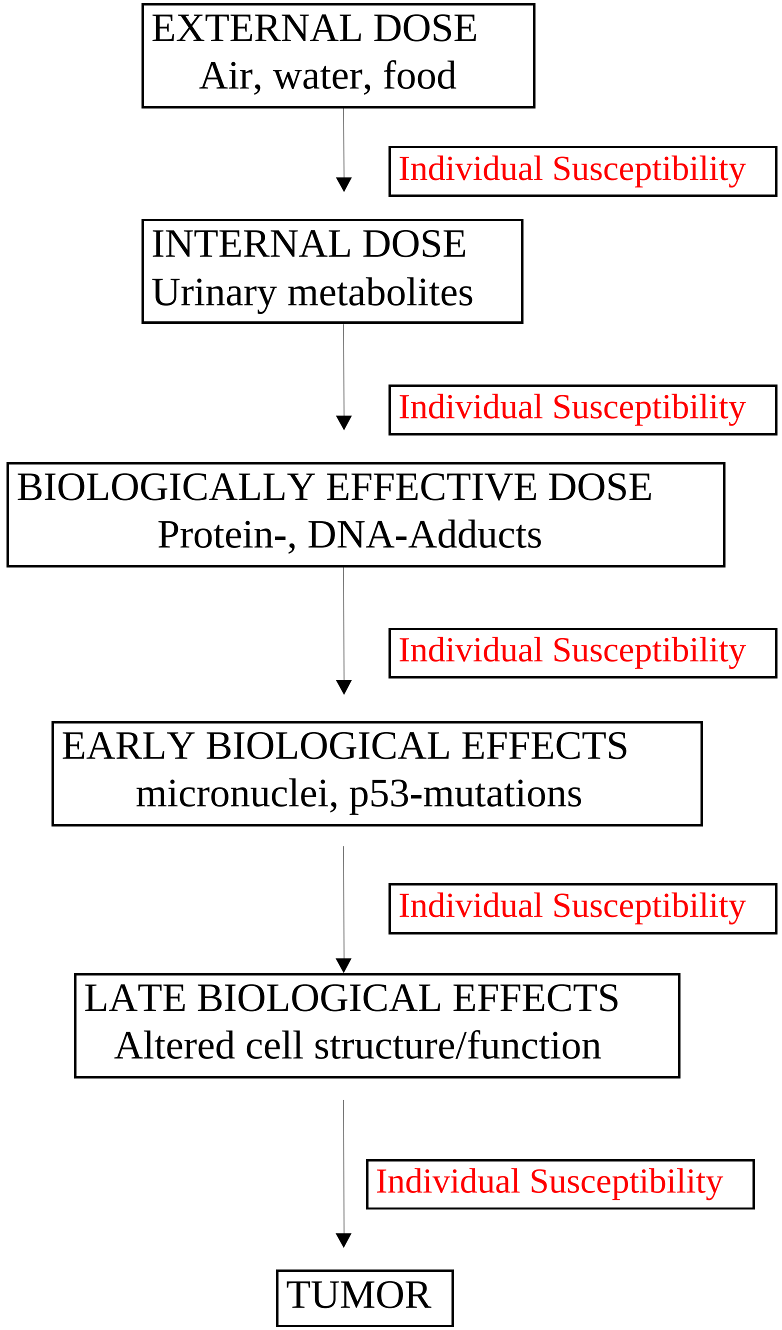
•The **applicability of the method for epidemiological studies will be applied to a group of 85 people**.

Lifetime' of Biomarkers

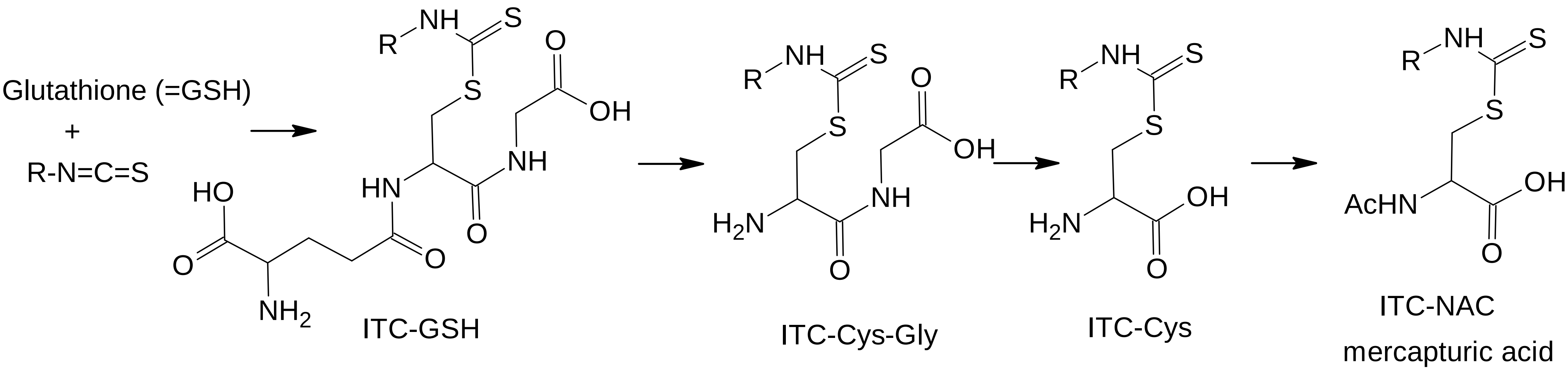


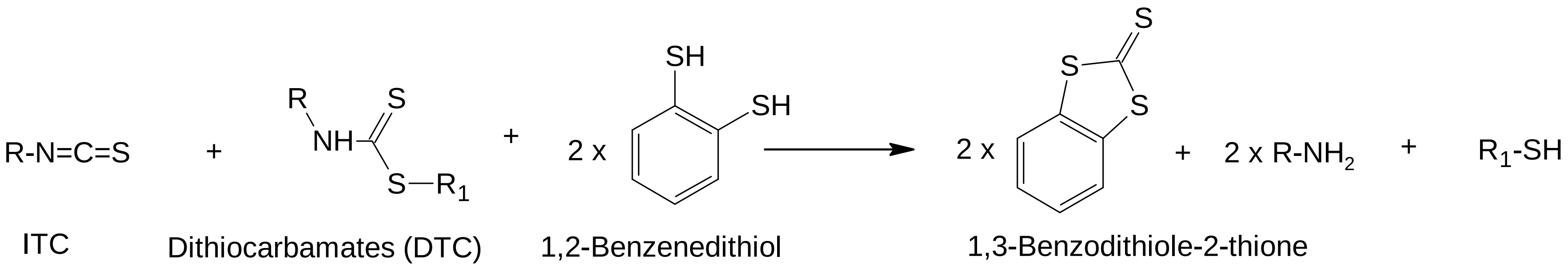
van Welie, et al (1992) Critit *Rev Toxicol*, **22**, 271-306. Henderson,et al(1989) *Crit Rev Toxicol*, **20**, 65-82.

The paradigm of biomonitoring



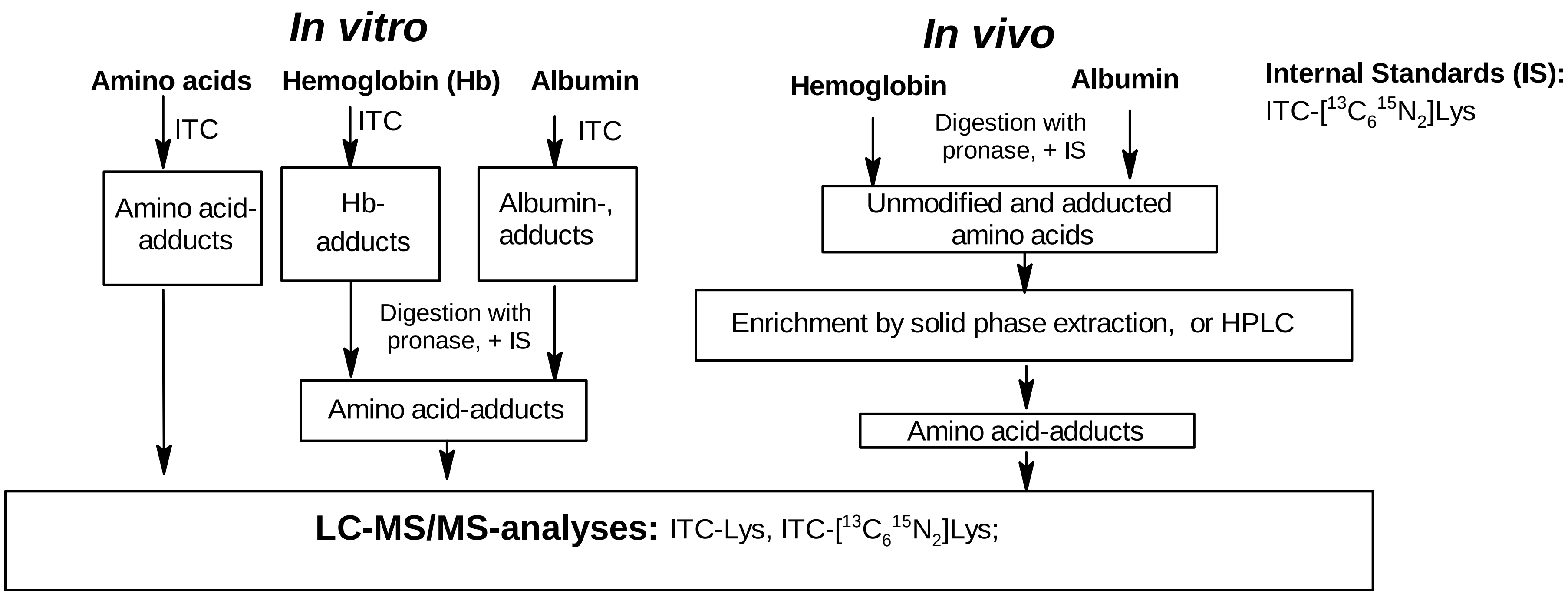
Urinary metabolites of Isothiocyanates





Reviewed in: Conaway et al (2002) *Curr. Drug Metab.*, ***3*, 233-55**

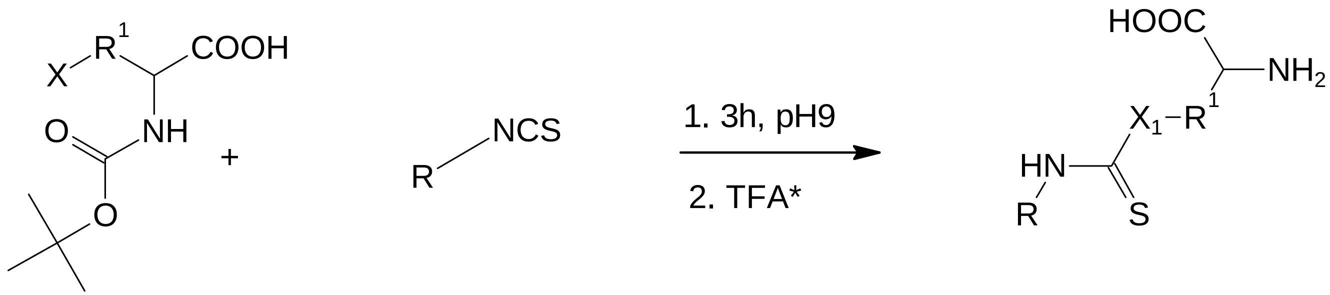
Research Design



Kumar, and Sabbioni (2010) *Chem Res Toxicol*, ***23, 756-65.***

Kumar, et al (2010) *Biomarkers*, ***15, 739-745.***

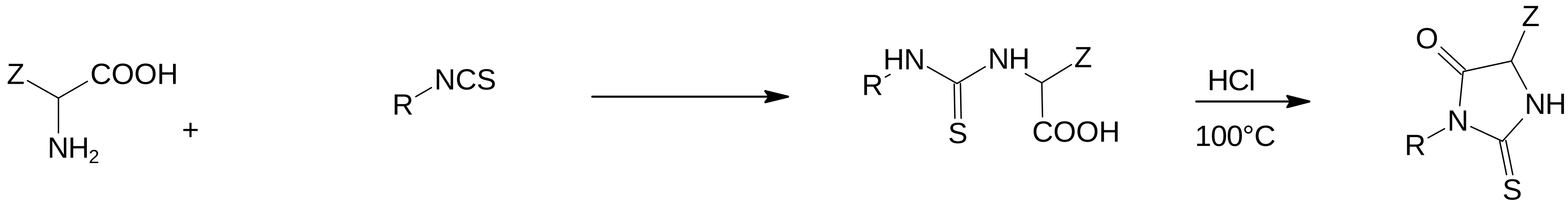
Synthesis: Reaction products of isothiocyanates with amino acids



N_α-Boc-Lys: $R^1=(CH_2)_4$; $X=NH_2$
N_α-Boc-Cys: $R^1=CH_2$; $X=SH$

BITC: R=Benzyl
PEITC: R=Phenethyl
AITC: R=Allyl
SFN:R=4-(methylsulfinyl) butane

BITC-Lys, PEITC-Lys, AITC-Lys, SFN-Lys:** X_1=NH
BITC-Cys,PEITC-Cys, AITC-Cys, SFN-Cys*:** X_1=S



Val: $Z=CH(CH_3)_2$
Asp: $Z=CH_2COOH$

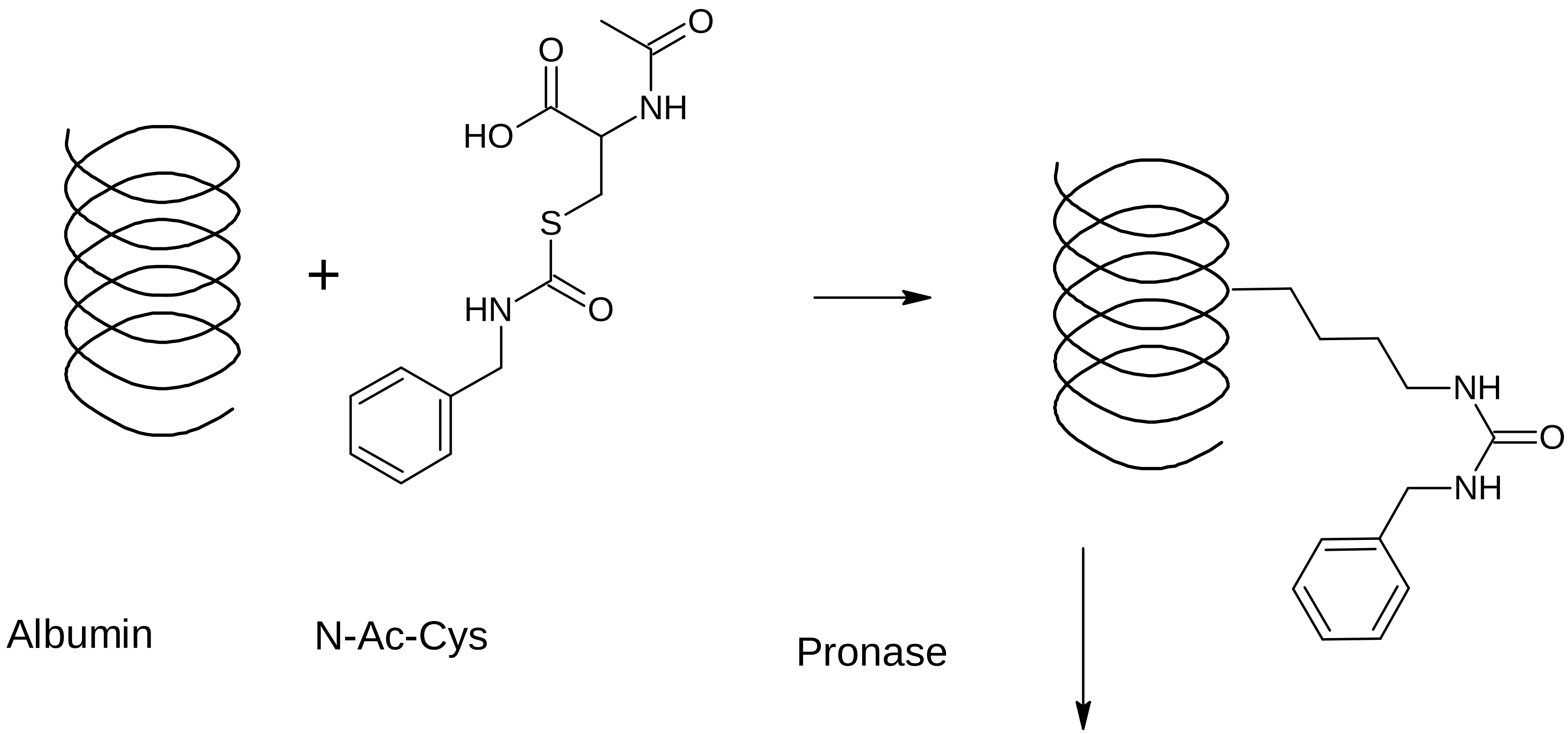
R=Benzyl

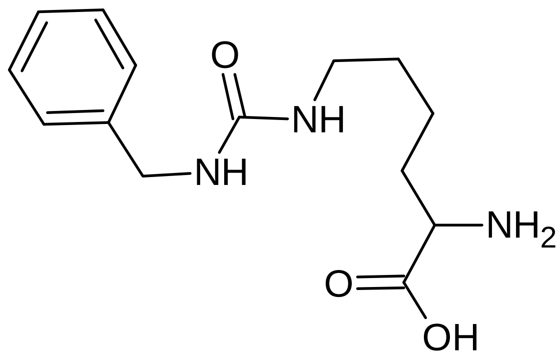
BITC-Val, -Asp,*

BITC-Val-Hyd, -Asp-Hyd,*

Kumar, and Sabbioni (2010) *Chem Res Toxicol*, ***23, 756-65.***

Isothiocyanate adducts with cysteine = Trojan Horse





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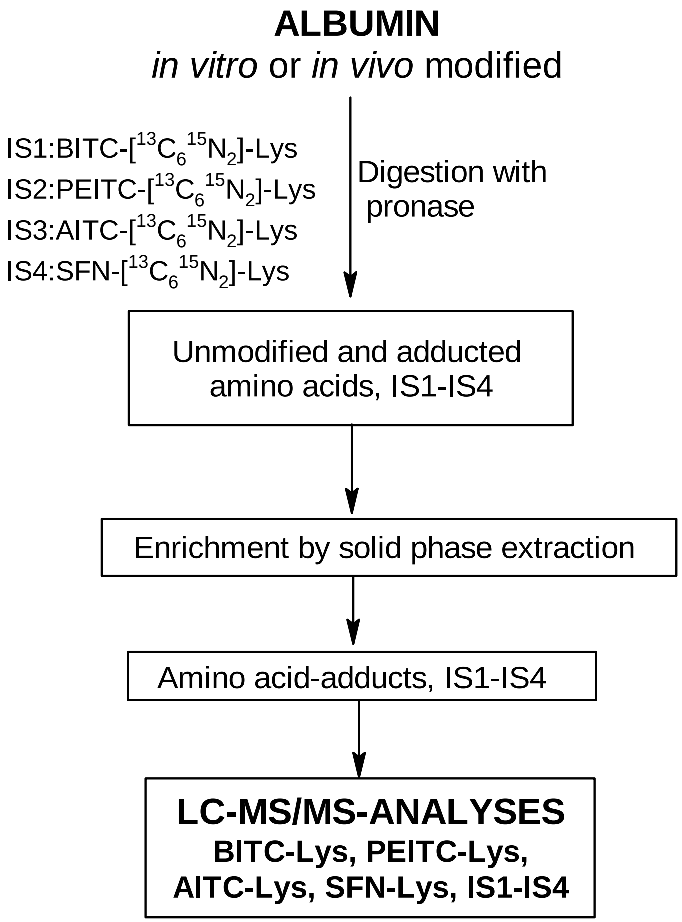
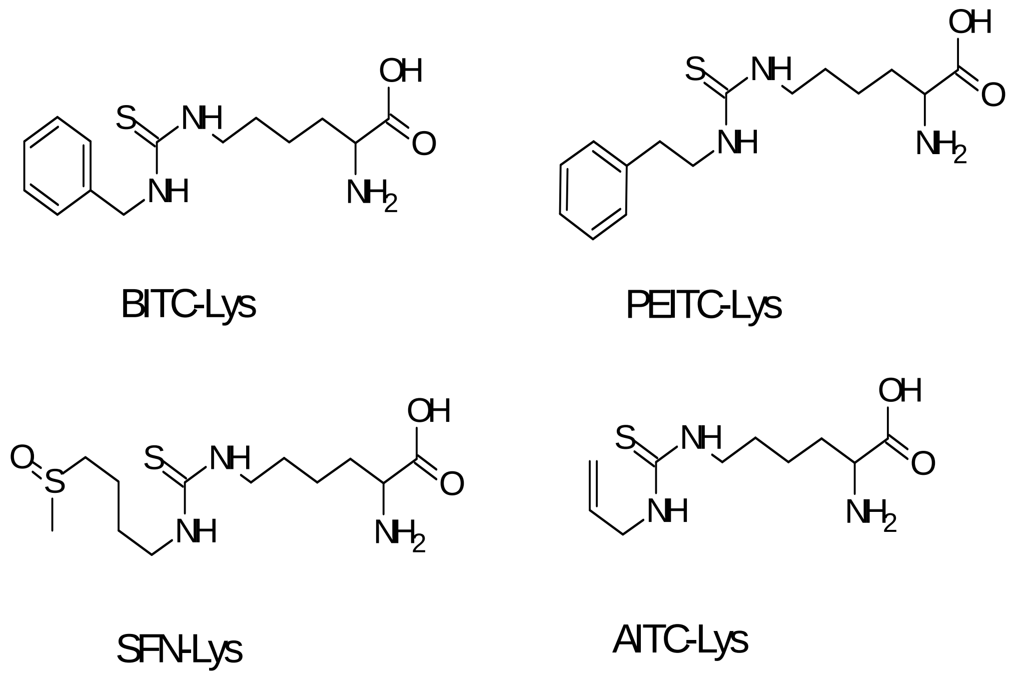
EXPERIMENTAL PROCEDURE
Determination of albumin adducts of isothiocyanates

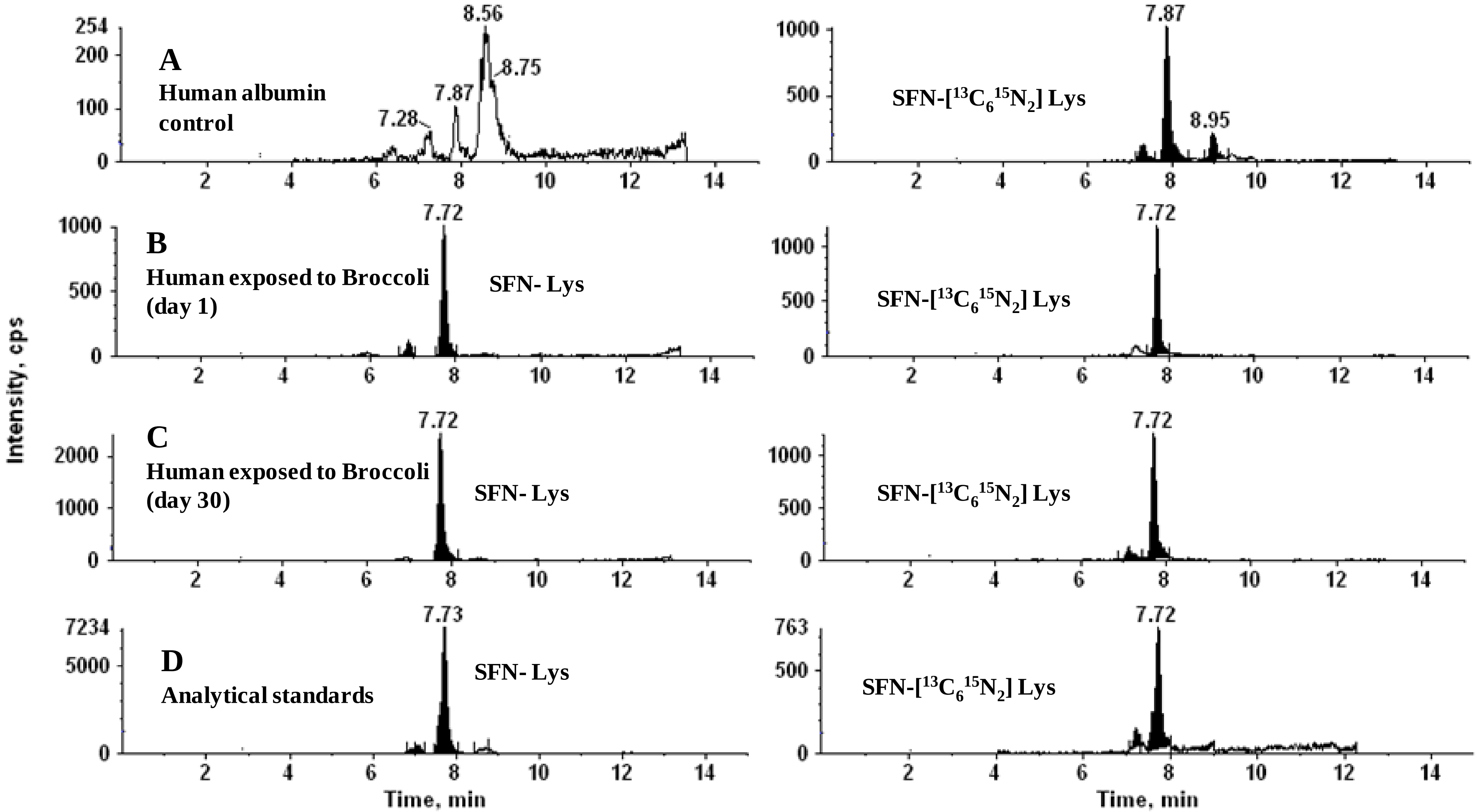




Kumar, and Sabbioni (2010) *Chem Res Toxicol*, ***23, 756-65.***
Kumar, et al (2010) *Biomarkers*, ***15, 739-745.***

EXPERIMENTAL PROCEDURE

LC-MS/MS analyses of the albumin adduct of SFN



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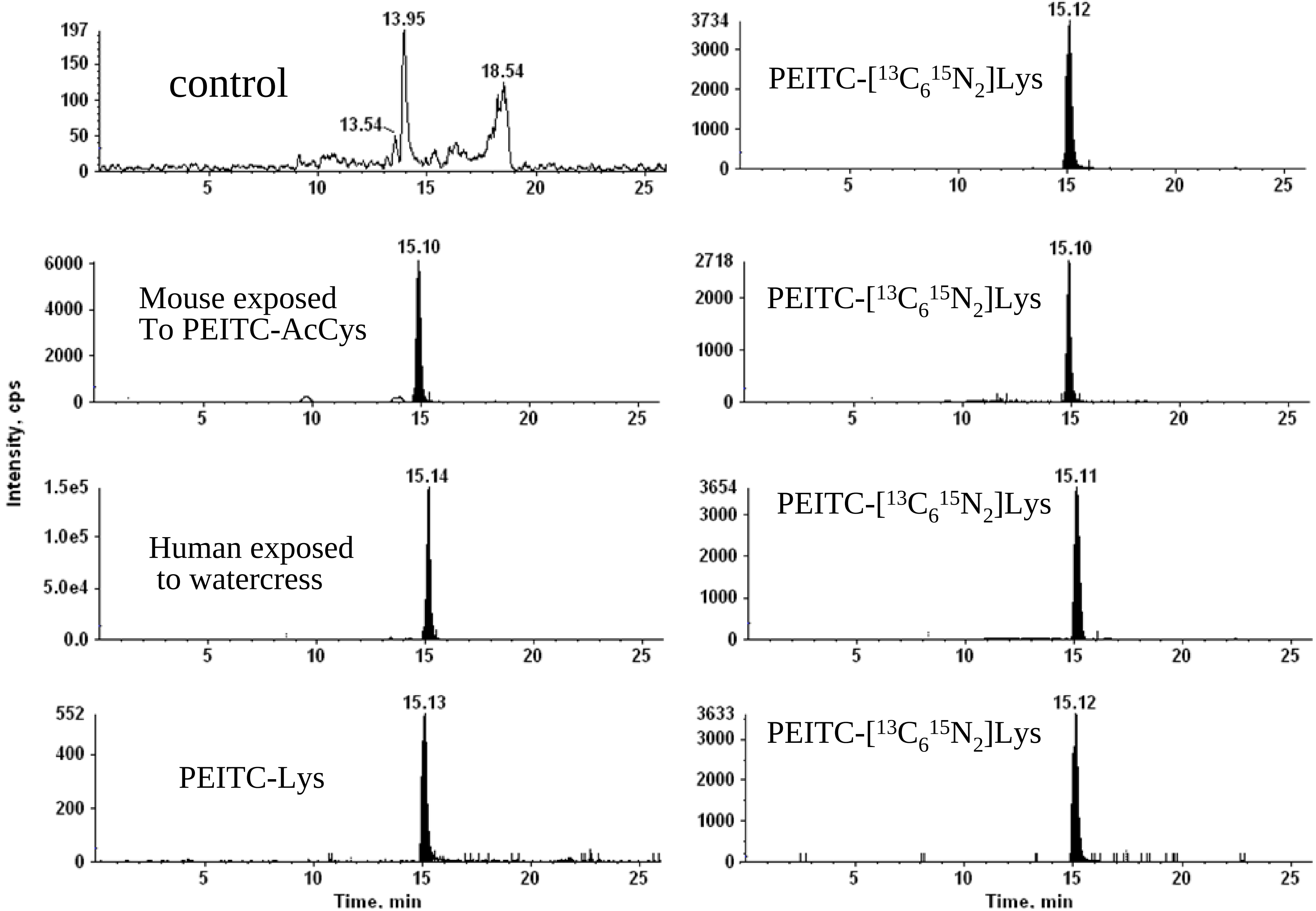


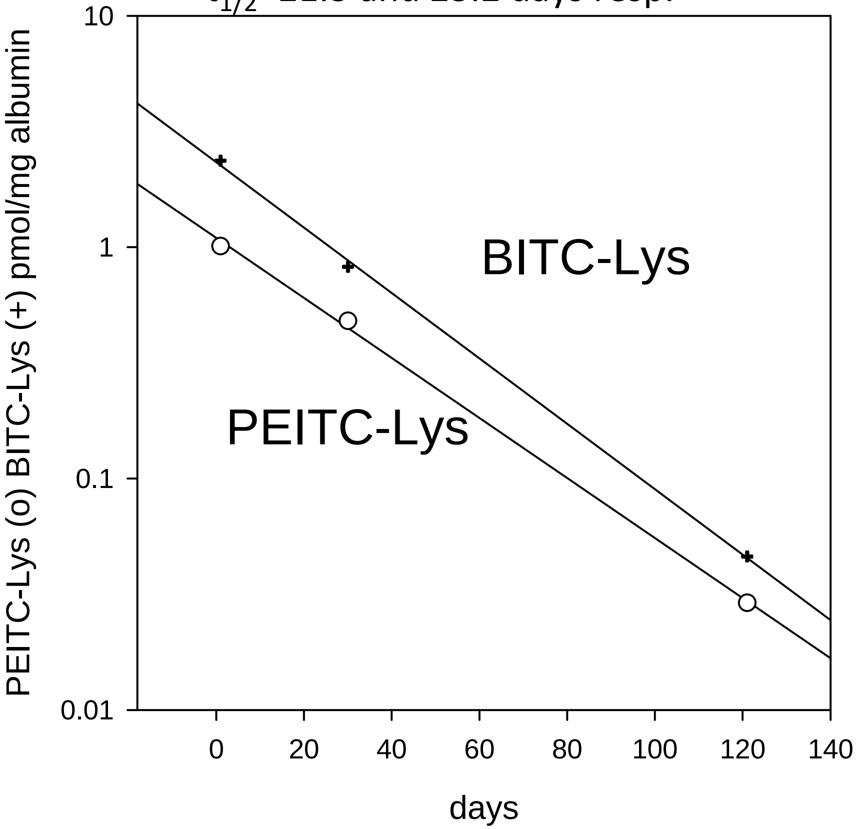
Kumar, and Sabbioni (2010) *Chem Res Toxicol*, ***23, 756-65.*** Kumar, et al (2010) *Biomarkers*, ***15, 739-745.***

RESULTS: Albumin (Alb) and hemoglobin (Hb) adducts of ITCs in a human subject eating garden cress and watercress on day 0 and eating regularly broccoli

Days	1	30	121
PEITC–Lys pmol/mg **Alb**	2.365	0.823	0.046
BITC–Lys pmol/mg **Alb**	1.011	0.480	0.029
SFN–Lys pmol/mg **Alb**	2.203	3.306	1.372
PEITC–Lys pmol/mg **Hb**	0.050	0.020	-[a)]
BITC–Lys pmol/mg **Hb**	0.023	0.021	-[a)]
SFN–Lys pmol/mg **Hb**	0.140	0.203	-[a)]

Albumin-adduct kinetics after a Single dose of BITC and/or PEITC

$t_{1/2}$=21.3 and 23.2 days resp.



[a)]Below limit of quantitation

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RESULTS: Albumin adducts of ITCs in a group of 85 Italian male smokers from Torino, Italy

The feasibility of the method was tested on biological samples collected previously for a diet-study. **The volunteers were randomly assigned to three groups**:

1. Group A (n=29): the diet was **rich in flavonoids**, but not supplemented.
2. Group B (n=29), the diet was a normal iso-caloric diet with an adequate administration of **fruit and vegetables**.
3. Group C (n=27): the diet was based on supplementation of the normal diet with **additional flavonoids** in the form of green tea and soy products.

Blood samples were collected at the beginning (0) and after 4 weeks (4).

After enzymatic digestion of albumin we determined the adducts of the isothiocyanates (ITC)s with lysine (Lys) using LC-MS/MS and isotope dilution method

Kumar, et al. (2010) *Biomarkers,* ***15, 739-745.***

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RESULTS

Albumin adduct levels of ITCs in the different diet groups

Set	Subjects (n)	AITC-Lys	**BITC-Lys**	PEITC-Lys	**SFN-Lys**
0	85	4.7%	**48%**	6%	**25%**
4 weeks	*85*	*2.4%*	***35%***	*11%*	***24%***

Correlation of the albumin adduct levels of ITCs after 4 weeks

	AITC-Lys-4	**BITC-Lys-4**	**PEITC-Lys-4**
BITC-Lys-4	0.238* (0.028)		
PEITC-Lys-4	0.474** (<0.001)	0.104 (0.342)	
SFN-Lys-4	0.328** (0.002)	0.205 (0.060)	0.480** (0.001)

Kumar, et al. (2010) *Biomarkers*, ***15, 739-745.***

Set	Group (n)	AITC-Lys	BITC-Lys	PEITC-Lys	SFN-Lys	BITC-Lys [fmol/mg] Median (25^{th},75^{th},90^{th})	SFN-Lys [fmol/mg] Median (25^{th},75^{th},90^{th})	Total cruciferous vegetables g/day Median (25^{th},75^{th},90^{th})
0	A n=29	3.4%	69%	10.3%	13.8%	5.39 [1,7)] (0, 7.07, 7.98)	0 [3)] (0,0, 71.7)	4.9 [8)] (3.5, 8.7, 12.2)
4	A n=29	6.9%	34.5%	17.2%	31%	0 [7)] (0, 6.18, 8.30)	0 (0, 25.2, 172)	68.8 [5)] (29.3, 110, 145)
0	B n=29	6.9%	55.2%	0%	17.2%	5.86 [2)] (0, 8.26, 12.7)	0 [4)] (0, 0, 98.9)	2.4 (n=17) [8)] (1.5, 8.9, 12.7)
4	B n=29	0%	37.9%	0%	10.3%	0 (0, 7.95, 11.3)	0 (0,0, 38.7)	6.3 (n=17) [5,6)] (3.0, 16.7, 32.7)
0	C n=27	3.7%	18.5%	7.4%	44.4%	0 [1,2)] (0, 0, 9.56)	0 [3,4)] (0, 43.3, 196)	4.5 (n=16) (2.0, 8.7, 18.1)
4	C n=27	0%	33.3%	14.8%	29.6%	0 (0, 5.74, 9.05)	0 (0, 21.3, 169)	38.7 (n=16) [6)] (26.5, 73.4, 109)

The levels of BITC-Lys and SFN-Lys and the total cruciferous vegetable levels determined with questionnaire were compared among the 3 diet groups and the two different time points (beginning of the study = set 0, and after 4 weeks= set 4). The significant differences found between 2 groups using the Mann-Whitney test were marked with a pair of equal numbers 1-8: [1-6)] $p < 0.05$; and [7,8)] $p < 0.1$. In addition the percentage of subjects found positive for all adducts (AITC-Lys, BITC-Lys, PEITC-Lys, SFN-Lys) were listed.

RESULTS

Correlation of the albumin adduct levels of ITCs after 4 weeks

	AITC-Lys-4	BITC-Lys-4	PEITC-Lys-4
BITC-Lys-4	0.238* (0.028)		
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RESULTS

Comparison of the diet with the albumin adduct levels of ITCs

Total cruciferous vegetables (4 weeks)	**AITC-Lys-0** 0.061 (0.639)	**BITC-Lys-0** -0.090 (0.485)	**BITC-Lys-0** 0.203 (0.114)	**SFN-Lys-0** 0.264* (0.038)
Total cruciferous vegetables (4 weeks)	**AITC-Lys-4** 0.306* (0.016)	**BITC-Lys-4** 0.190 (0.14)	**PEITC-Lys-4** 0.403** (0.001)	**SFN-Lys-4** 0.399** (0.001)

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CONCLUSIONS

1.Isothiocyanates=ITCs (AITC, PEITC, BITC, SFN) react with lysine present in proteins such as albumin: **ITC-Lys**

2.Using LC–MS/MS, ITC–Lys could be determined in up to half of the study subjects.

3.Since cell **protein adducts are involved in the chemopreventive effects** of ITCs, blood protein adducts are probably not only **a biomarker of exposure but also a potential surrogate marker** for the effects of ITCs at the cellular level.

4.This new method will enable to quantify **ITC adducts in blood proteins** from **large prospective studies about diet and cancer**, overcoming the limitations of questionnaire estimates.

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