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# Influence of heat treatments on sulforaphane in italian *brassica* cultivars

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**Titolo**
Influenza dei trattamenti termici sul sulforafane nelle cultivar di *brassica* coltivate in Italia

**Key words**
Broccoli, cauliflowers, cabbage, cooking methods (microwave, boiling water, steam, pan cooking), sulforaphane; GC/MS analysis

**Parole chiave**
Broccoli, cavolfiore, cavolo, metodi di cottura (microonde, acqua bollente, vapore, frittura), sulforafane, analisi GC/MS

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**Summary**
Organic isothiocyanates (ITCs), and particularly sulforaphane, are widely studied as potential inhibitors carcinogenesis in animal models including cancer of lung, esophagus, forestomach, colon, mammary glands and pancreas. Sulforaphane, enzymatic breakdown product of glucoraphanin, was extracted by solvent from vegetable homogenates and quali-quantitatively analysed by GC/MS. This method, modified from VanEtten procedure, was used to evaluate sulforaphane in fresh and cooked (microwave, boiling water, steam, pan cooking) broccoli, cauliflower ("Romanesco", "verde di Macerata", "violetto di Catania", "Jesino" and commercial hybrid) and white cabbage. The highest content in sulforaphane was noted in the raw broccoli, respect of the other examinated fresh *brassica* cultivars. When all these samples were cooked, the retention of sulforaphane, the most potent activator of phase II enzymes among all ITCs, were higher in the microwave cooked samples (61-86%), little lower in the boiling water (38-74%) and strongly lower in the steam (18-52%) and pan cooking (14-48%). The microwave seems to be the most efficient cooking method for all these vegetables because it shows the highest retention in sulforaphane.

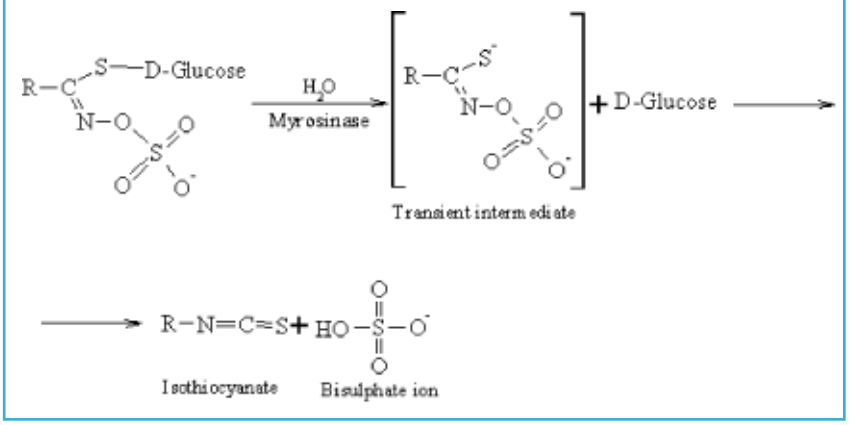
**Riassunto**
Gli isotiocianati, ed in particolare il sulforafane, sono largamente studiati in quanto hanno la capacità di inibire alcune forme tumorali che interessano la lingua, esofago, stomaco, colon, ghiandole mammarie e pancreas, in animali da laboratorio e nell'uomo. Il sulforafane, prodotto di idrolisi enzimatica delle glucorafanina, è stato estratto con solventi dai vegetali omogeneizzati ed analizzato quali-quantitativamente per via GC/MS. Questo metodo, ottenuto in seguito a modifiche di quello originale di VanEtten, è stato usato per la determinazione del sulforafane nei campioni freschi e cotti (microonde, acqua bollente, vapore, frittura) di broccolo, cavolfiore ("Romanesco", "verde di Macerata", "violetto di Catania", "Jesino" ed un ibrido commerciale) e cavolo-cappuccio bianco. Nei campioni freschi di broccolo è stato accertato il più alto contenuto in sulforafane, rispetto alle altre cultivar allo stato fresco. Quando tutti i campioni sono stati cotti, la ritenzione percentuale del sulforafane, il più potente

attivatore degli enzimi della fase II tra tutti gli isotiocianati, è stata più elevata nei campioni cotti con microonde (61-86 %), un po' più bassa nella cottura in acqua bollente (38-74 %) ed ancora più bassa nella cottura a vapore (18-52 %) e frittura (14-48 %). Le microonde si sono dimostrate il più efficiente sistema di cottura per questi vegetali, poiché esso mostra le più alte ritenzioni in sulforafane per tutti i vegetali presi in esame.

**Introduction**

The natural organic isothiocyanates (ITCs) are synthesized and stored as glucosinolates (β-thioglucoside N-hydroxy sulfates) in cruciferous vegetables consumed by humans, such as broccoli, cabbage, cauliflower, radish, turnip and watercress (1-3).
The glucosinolates are hydrolyzeed to isothiocyanates by myrosinase (β-thioglucoside glucohydrolase; EC 3,2. 3-1), that is normally segregated from glucosinolates and is released after cellular damage. Myrosynase catalyzes at pH=7 the following reaction (4, 5) (Fig. 1).
It has long been known that ITCs have different biological effects due to their chemical reactivity (2, 6, 7). More recently, ITCs such as 4-methylsulfinylbutyl NCS or sulforaphane, 3-methylsulfinylpropyl NCS, 3(methylthio)propyl NCS, 2-phenylethyl NCS, allyl NCS and methyl NCS (8, 9), have been studied for their effects in cancer prevention. These ITCs are potent inhibitors of tumorigenesis in various animal models (10, 11, 12). The dual action of ITCs is to inhibit phase I enzymes that are responsible for the bioactivation of carcinogenesis, reducing the production of electrophilic intermediates (13) and to enhance the activity of phase II enzymes such as glutathione transferases, epoxide hydrolases, NAD(P)H: quinone reductases and glucoronosyl transferases, that increase the detoxification and clearance of carcinogens (14-16).
The effects on phase I and II enzymes, and consequently a reduced cancer risk, have been demonstrated in humans that consume a large quantity of cruciferous vegetables (12, 17-22).
Recent works have also shown that ITCs act as suppressing agents during the promotion of neoplastic process and as signal transduction pathways within the cell, induce apoptosis and inhibit cell grown (23-26).

**Figura 1** - Production of isothiocyanates from glucosinolates



A large number of *brassica* vegetables are consumed after cooking and the amounts of glucosinolates, and consequently of the corrispective isothiocyanates, are usually reduced in the cooked vegetables (27).

Jiao et al (28) determined the content in total isothiocyanates in 102 cooked vegetables consumed in Singapore. They used an HPLC method to quantify the cyclic products of ITCs and 1,2-benzenedithiol after treatment of vegetable juices with myrosinase. The content of total ITCs in 9 type of cruciferous vegetables ranged between 4.9 μmol 100 $g^{-1}$ d.m. in bokchoi and 81.3 μmol 100 $g^{-1}$ d.m. in watercress.

Wennberg et al (29) studied the effect of blanching and treatment with white vinegar on glucosinolates of white cabbage (cv Heckla, Predikant). Glucosinolates content decreased substancially in both blanched cultivars, although the total loss was higher in Predikant (74%) than in Heckla (50%). The individual glucosinolates were effected to different degrees (15-91%). During souring with acetic acid, total content of glucosinolate was not effected in Heckla but was further reduced in Predikant. A substantial increase in 4-methoxyglucobrassicin was noted in both cultivars.

Rosa et al (30) measured the concentrations of individual and total glucosinolates in four type of portuguese cabbage and in one hybrid of white cabbage before and after cooking for 5-10 min in boiling water. Analysis of fresh cabbage, cooked leaves and cooking water showed that the glucosinolates content of cabbages was reduced by more than 50%. Almost all of this loss was accounted as intact glucosinolates in the cooking water. Even in Italy a lot of papers were published about the content of glucosinolates in fresh crucifers (31-33).

The aim of this paper is to evaluate the influence of different heat treatments (microwave, boiling water, steam and pan cooking) on the sulforaphane content in some *brassica* cultivar (broccoli, cabbage, cauliflower) that are frequently consumed in Italy.

In the present research, to determinate the sulforaphane content in the *brassica* samples it was used a GC/MS procedure, modifying the original method of VanEtten (34, 35). Sulforaphane was also determined with a similar procedures by Chang et al (36) and Omary et al (37).

## Material and methods

### *Plant source*

Cauliflower "Romanesco", "verde di Macerata", "violetto di Catania", "Jesino" and commercial hybrid (*Brassica oleracea* var. Botrytis) were grown in the C.R.A.-ISOR, Monsampolo del Tronto (AP) fields.

Broccoli (*Brassica oleracea* var. Italica) and white cabbage (*Brassica oleracea* var. Capitata) were purchased at the Milan general *horticultural* market.

The heads of broccoli and cauliflowers were decored to florets before using. The heads of white cabbage were cut as a wedge with the edge parallel to the core. Than the samples were cut into 3-5 cm cube before using.

### *Cooking methods*

a) Microwave: 300 g of each sample were cooked in 1L special glass becker, containing 800 mL of water with 0.3% of NaCl, covered by a Petri glass. Microwave power applied was 900-1000 watt for 5-6 min for all samples.
b) Boiling water: 300 g of samples were cooked for 6-8 min in 1300 mL of boiling water with 0.3% of NaCl for all samples.
c) Steam: 300 g of each sample were cooked in a steam cooker Multigourmet (Braun) for 20-25 min, for all sample. In the lying container were put 1500 mL of water.
d) Pan cooking: 300 g of each sample were cooked in steel pan with 15-20 g of olive oil and 3 g of NaCl for 18-22 min.

For all the sample, all the tests were repeated three times.

Isolation and purification of glucoraphanin from broccoli seeds.

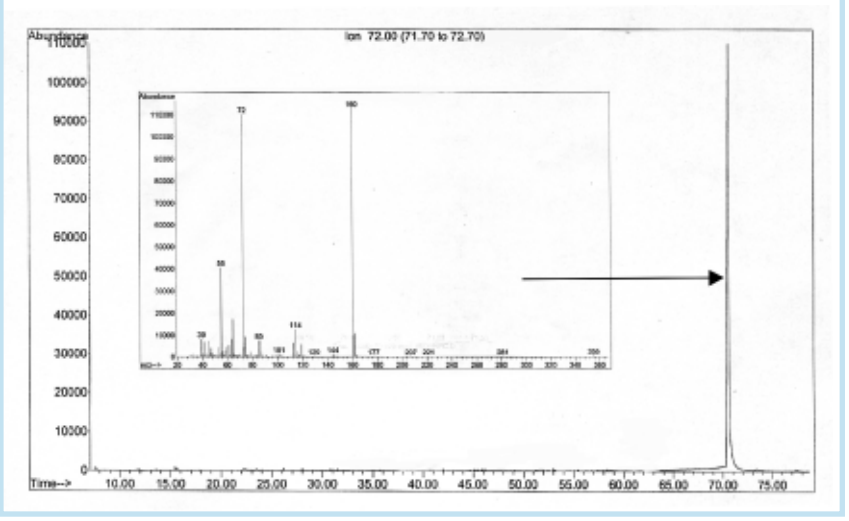
Glucoraphanin was prepared from broccoli seeds with boiling water, purified and concentrated by SPE and preparative scale HPLC, according to Rochfort et al (38), using method 3 for the concentration of the glucoraphanin.

To evaluate the purity of glucoraphanin, the aqueous fraction was submitted to modified method, following described, for determination of relative breakdown sulforaphane, that was quali-quantitatively analysed by GC/MS. The chromatogram in figure 2 shows that the substance content in the fraction with 22-25 min of retention time is glucoraphanin, because its breakdown product is sulforaphane. In fact the mass spectrum is characteristic of sulforaphane compounds and the retention time is the same of the commercial standard.

GC/MS determination of isothiocyanates as breakdown products of glucosinolates by VanEtten et al. (original method)

The extraction of glucosinolates from *brassica* samples, their absorption on the anion exchange resin (DOWEX1x2), their hydrolysis with aqueous myrosinase preparation (39) to isothiocyanates in methylene chloride and finally the quali-quantitative analysis of isothiocyanate by GC/MS was performed according to VanEtten et al (34, 35).

**Figure 2** - GC/MS chromatogram of sulforaphane obtained from hydrolysis of glucoraphanin prepared according to Rochfort et al (31). The mass-spectrum of sulforaphane was in agreement with previously published by Spencer at al (39)




*Determination of sulforaphane (modified method)*

15 g of fresh or 1.5 g of frozen dry samples were homogenized with 50 mL of boiling methanol. The mixture was boiled for 15 min in a flask covered with a watch glass. After cooling, the mixture was centrifuged at 4000 rpm for 10 min and than filtered on glass wool. The residue was dispersed in 30 mL of 70:30 methanol:water solution, homogenized for 5 min and than performed as described hereafter.

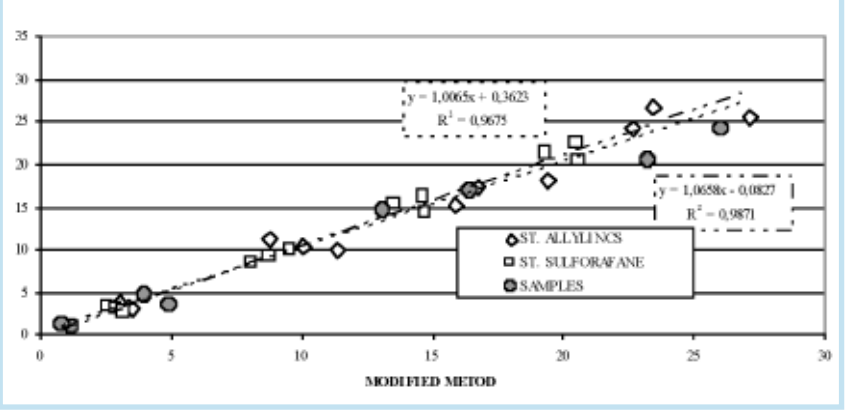
The combined filtrates were reduced to about 10 mL under vacuum rotavapor at T< 60°C. The concentrate was centrifuged at 4000 rpm for 10 min and the supernatant diluted to 25 mL with distilled water.

Enzymatic conversion of glucoraphanin in sulforaphane was done in a 100 ml teflon tube, adding 12.5 mL of phosphate buffer solution pH=7 (30.5 ml of $Na_2HPO_4 \cdot 2H_2O$ 0.2 M and 19.5 mL of $NaH_2PO_4 \cdot 1H_2O$ 0.2 M were diluted to 100 mL with distilled water) and 40 mg of thioglucosidase (E.C. 3.2.3.1. Sigma Aldrich, Milano) to 12.5 mL of homogenate.

The tube was tapped and shacked for 60 min horizontally in a

box–typer shaker at 80-100 oscillations for minute at room temperature. The mixture was added of 25 mL of methylene chloride, shacked for 5 min and centrifuged for 10 min at 4000 rpm.
The methylene chloride fraction was filtered through a paper filter in a 250 mL glass flask. The homogenate was extracted a second time as described above. Both methylene chloride fractions were pooled, dried over anidrous $Na_2SO_4$ and then filtered trough a paper filter. The filtrate was concentrated until 1 mL under reduced pressure at 35°C using a rotary evaporator. The methylene chloride extract was injected into GC/MS (6890 N network GC system; 5973Mass Selective detector, Agilent Technologies).
For the qualitative analysis, 1 µL of the extract was injected at 200°C into a DB-1 capillary column (60m x 0.25 mm i.d., 0.25 µL film thickness, J & W Scientific, Folsom, CA). The column temperature was kept at 50°C for 5 min and than raised to 240°C at 2°C/min heating rate; finally it was kept at 240°C for 20 min. The inlet flow and the split of the carrier (He) were set at 2 mL/min and 10 mL/min, respectively. The temperature of the transfer-line was 240°C. The MS spectra was generated at 70 eV, and the 10-400 Amu mass range was selected for mean square displacement (MSD). Compounds were identified by comparing spectra of standard and spectra contained in the instrument library, or by comparing retention time of standard.
The calculation of sulforaphane and allyl NCS concentrations were based on the response factor, using solutions standard of sulforaphane and allyl NCS (SIGMA) to encompass three series of concentrations.

**Figure 3** - Regression curve obtained by comparison VanEtten and modified methods, using standard (ST) solutions of Allyl NCS, sulforaphane and *brassica* extracts (SAMPLES).
– – – – = regression curve of Allyl NCS;
— – – – — = regression curve of sulforaphane.
Data are expressed as µmol $g^{-1}$.




*Methods comparison*

The modified method was compared with the VanEtten method using four standard solutions of glucoraphanin, (2.95-20.63 µmol $g^{-1}$, prepared following the Rochfort et al (38) method), sinigrin (3.37-23.61 µmol $g^{-1}$, purchased from SIGMA) and some fresh *brassica* samples (broccoli, cauliflower "Romanesco", "violetto di Catania" and "verde di Macerata"). In figure 3 is reported the regression curve of breakdown products of glucoraphanin (sulforaphane), raw *brassica* samples (sulforaphane) and sinigrin (allyl NCS). Linear regression of concentrations of standard solution and raw samples obtained with two methods give a correlation coefficient of 0.9675 for allyl NCS and 0.9871 for sulforaphane both in the standard solution and in the *brassica* samples. In the modified method, the quantities of starting samples and solvents (methanol, methylene chloride) are reduced and the

anion exchange resin is not used to separate glucosinolates from free sugars to the original method. For our purposes is not important to know the content of total glucosinolates, determined by the glucose hydrolyzed from glucosinolates. Besides, to establish the optimum yield of sulforaphane the enzyme hydrolysis of relative glucosinolate is performed for various time (15, 30, 60, 120 min) at pH=7 and room temperature on a pool of examined raw extracts. Figure 4 shows that the optimum yield is obtained after 30-60 min of incubation.

For the determination of reproducibility, a sample of each raw cultivar is extracted and analysed four times. The relative standard deviation of sulforaphane concentration ranged between 4.91 and 7.25%.

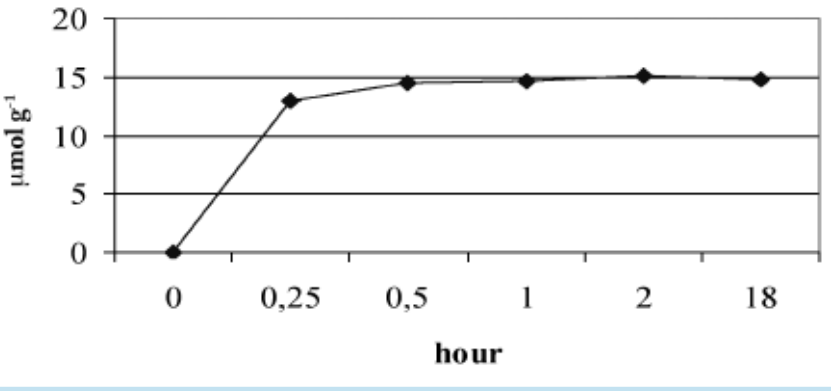
*Dry matter*

Four samples of each cultivar, raw and treated, were dehydrated in a laboratory oven kept at 85°C until a constant weight. The moisture content was calculated from the difference between the wet and the dry weight divided for the wet weight (40).

*Statistical analysis*

The Tukey HSD test was used to evaluate the difference between fresh and treated samples. Mean values were considered significantly different when $\rho \leq 0.05$.

**Figure 4** - Time course of sulforaphane formation when 12,5 mL of extract are incubated with 40 mg of thioglucosidase and 12,5 mL of phosphate buffer solution pH=7 at room temperature. Mean values of three replicates for each solution




**Results**

Among the Italian *brassica* cultivars, the higher levels of sulforaphane is found in fresh broccoli (25 µmol $g^{-1}$) (Tab. 1), while in the cauliflower "Jesino", white cabbage, cauliflower "verde di Macerata" and white cauliflower commercial hybrid show the lowest values, ranged among 0.25-1.60 µmol $g^{-1}$. Levels between 4-15 µmol $g^{-1}$ are respectively ascertained in the cauliflower "violetto di Catania" and "Romanesco". From the same table it is noted that all the cooking methods cause a decrease on sulforaphane content in all the examined cultivars respect of the raw vegetables. These decreases depend on the cooking method used and on the type of cultivar. The sulforaphane quantity retained into the vegetables after cooking, is expressed as % retention.

In the figure 5 are reported the % retentions of sulforaphane in all the treated vegetables. The samples cooked by microwave have the highest retentions respect of the other cooking methods, except the cauliflower "verde di Macerata", where the retention of Mw and boiling water cooking are similar.

The retentions of boiling water are superior than the other methods in broccoli, cauliflower "violetto di Catania", "verde di Macerata" and white cabbage commercial hybrid.

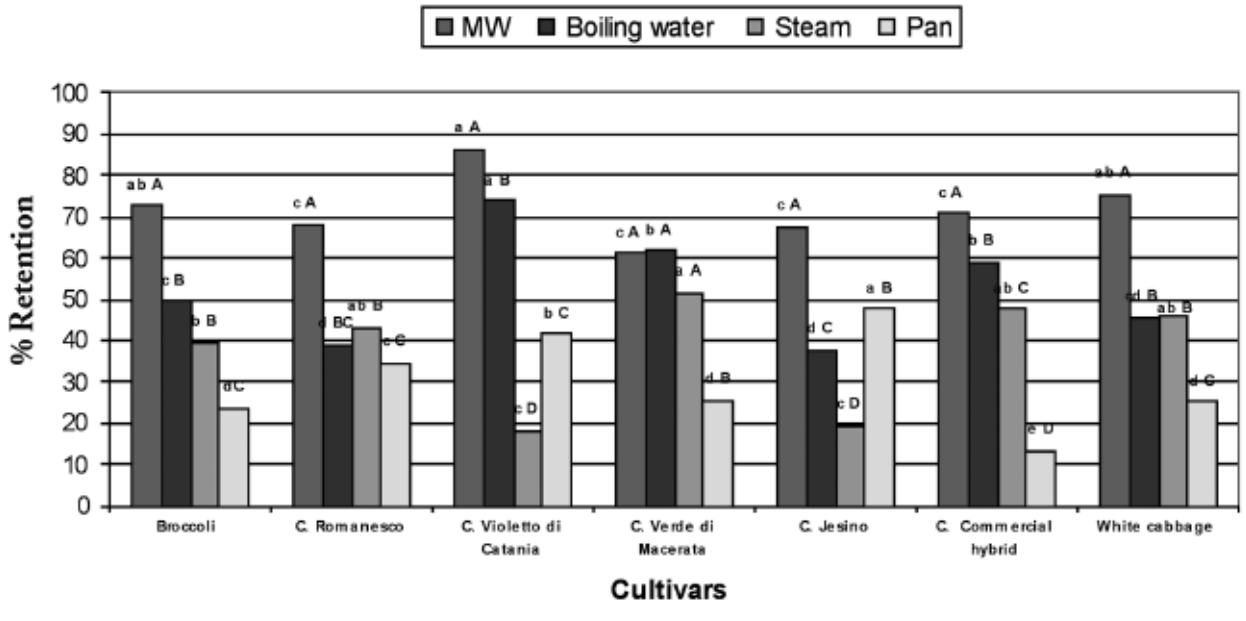
The retention of boiling water are similar in white cabbage cooked by steam, inferior in cauliflower

**Table 1** - Sulforaphane content (µmol g-1 d.m.) in raw and cooked cultivars. Mean values of three replicates for each cultivar.

| | Raw | Mw | Boiling water | Steam | Pan |
|---|---|---|---|---|---|
| Broccoli | 24.62 a A | 17.98 a B | 12.26 a C | 9.87 a C | 5.93 a D |
| C. Romanesco | 14.76 b A | 10.13 b B | 5.77 b C | 6.42 b C | 5.17 a C |
| C. violetto di Catania | 4.44 c A | 3.84 c AB | 3.33 c B | 0.82 c D | 1.84 b C |
| C. verde di Macerata | 1.02 de A | 0.61 d B | 0.62 d B | 0.52 c B | 0.26 c C |
| C. Jesino | 0.25 e A | 0.17 d B | 0.09 d C | 0.05 d D | 0.12 c C |
| C. Commercial hybrid | 1.60 d A | 1.14 d B | 0.95 d C | 0.77 c D | 0.22 c E |
| White cabbage | 0.89 de A | 0.67 d B | 0.41 d C | 0.41 c C | 0.23 c C |

Small letters indicate statistically significant difference among cultivars in the same cooking method (ρ≤0,05). Capital letters indicate statistically significant difference among cooking methods in the same cultivar (ρ≤0,05). C = cauliflower

**Figure 5** - % retention of sulforaphane in the cooked samples. Mean values of three replicates for each cultivar. Small letters indicate statistically significant difference among cultivars in the same cooking method (ρ≤0,05). Capital letters indicate statistically significant difference among cooking methods in the same cultivar (ρ≤0,05). C = cauliflower



"Romanesco" cooked by steam and cauliflower "Jesino" by pan-cooking.
The retentions of the steam are higher respect of pan cooking in broccoli, cauliflower "Romanesco", "verde di Macerata", commercial hybrid and white cabbage. While they are inferior only in cauliflower "violetto di Catania" and "Jesino" by pan-cooking.
So, when the vegetables are cooked, we can see that in Mw the retentions of sulforaphane content are the highest as concern all the cultivars and range 61-86%. The retentions of sulforaphane content decrease between 38-74% in the boiling water, lower values are showed respectively in steam (18-52%) and pan cooking (14-48%).

**Discussion**

The most of sulforaphane content decrease in all the examinated *brassica* cultivars, when the vegetables are submitted to cooking treatments.
As ITCs are breakdown products of glucosinolates, the decrease of ITCs depended on the corrispective decrease of glucosinolates. Losses of glucosinolates were reported in previous studies by Sones et al (41). They noted that in cabbage and cauliflower cooked in boiling water for 10 min, glucosinolates were reduced about 20-30%.
Glucosinolates are relatively stable at high temperature (≤100°C) and water soluble (29). Microwave, boiling water and steam cooking are performed around 100°C. In this condition, glucosinolates are stable and the decrease of glucosinolates and consequently of sulforaphane is due to removal of intact glucosinolates from vegetable tissues in the cooking water. This behaviour is according to Slominski et al (42). They demonstrated that 90% of intact glucosinolates or their breakdown products, were found in the vegetable tissues (40%) and in the cooking water (50%), when the cabbage was cooked in water for 40 min. The explanations of the differences in losses of sulforaphane and other ITCs after heat treatments, could depend on the cultivar, leaf thickness and waxes, size of florets, fibre content, diffusivity of the glucosinolates in vegetable tissues (Rosa et al, Sones et al) (24, 41). The loss of glucosinolates from vegetable depends on the quantity of the processing water. Higher is the quantity of water and higher is their losses. In our experiments the quantity of water used for microwave (800 mL) is smaller respect of the boiling water (1300 mL) and steam (1500 mL). This can explain the better recoveries of sulforaphane during Mw cooking. In the pan cooking, the temperature is higher (160-180°C) for the presence of olive oil. In this case, the high losses of sulforaphane can be also depended on the thermal degradation of glucoraphanin. Oerlemans et al (43) studied the thermal degradation of glucosinolates in red cabbage submitted to three different heat treatments (blanching, cooking and canning). They noted that the glucosinolates are not influenced during blanching (3 min at 95°C); while its losses during boiling water cooking (40 min at 100°C) and canning (40 min at 120°C) were respectively 10 and 85%. However, some ITCs such as sulforaphane in the first step of cooking, may escape into the air or degrade to the volatile sulphur compounds (44). Recent researches have demonstred indigenous myrosinase activity in the gut microflora (45). However, little is known about the amounts of ITCs produced by the human microflora from glucosinolates. Krul et al (45), in an intetstinal model system with a pooled and culturated human microflora, found that the conversion of sinigrin into allyl NCS ranged between 10 and 30% and allyl NCS is converted further in other unknown metabolites.
More recently, Iori et al (46) have studied the in vitro gastrointestinal digestion of broccoli, using as evaluation index glucosinolates, phenolic compounds and vitamin C.

## Conclusions

Brassica cultivars are of great interest in the field of human nutrition and disease prevention. These capacities are due to the ITCs, such as sulforaphane, produced by the enzymatic hydrolysis of glucoraphanin. The used GC/MS method, is sensitive, reproducible and suitable for routine analysis and is applied to determine sulforaphane in some fresh and treated brassica cultivars. There is a substantial leaching of sulforaphane during cooking processes. If the heat treatment is performed around 100°C as boiling water, steam and microwave cooking, almost all these losses are accounted for as intact glucosinolates in cooking water. Above 100°C (pan cooking) its losses can depend on the thermal degradation. Among the cooking methods, microwave is thought to be an efficient alternative for cooking these vegetables due to the low amount of cooking water required and shorter cooking times. The cooking water can be used as base in soup or gravy, so that glucoraphanin or its breakdown product (sulforaphane) may enter the body with the part remaining in the cooked vegetables.

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