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Comparative Fingerprint of Glucosinolates from *Brassica* Vegetables Using HATR/FT-MIR Spectroscopy

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Abstract. Glucosinolates (β-thioglucoside-N-hydroxysulphates) are very important plant secondary metabolites containing a β-D-glucopyranose residue linked to a sulfur atom (i), or to a (Z)-N-hydroximinosulfate ester (ii) or a variable R group (iii) with different side-chain substituents which give their structural diversity.

The objective of the present research was to compare the fingerprint of 9 standard pure glucosinolates and *Brassica* vegetables extracts of broccoli, cauliflower and kohlrabi, cultivated in North-West of Romania, using the Fourier Transform Infrared Spectroscopy (FTIR). The FTIR fingerprint was recorded for both, desulpho – standards of glucosinolates (sinigrin, gluconapin, progoitrin, glucoiberin, glucoraphanin, glucotropaeolin and glucobrassicin) and intact glucosinolates (sinigrin and glucotropaeolin). The extraction of glucosinolates was made according to the EU official method (EEC Regulation N. 1864/90). The FTIR spectra for standards and *Brassica* dried extracts were recorded in the MIR region, from 4000 and 900 cm^{-1}.

The results obtained show that HATR/FT-MIR spectroscopy can be used as a fast method for the quantification of total glucosinolates considering the IR absorption at 800 cm^{-1}or the region area of 750-900 cm^{-1}, is the most suitable. The HATR/FT-MIR is non-destructive, cheap and fast method to fingerprint, to predict and to quantify the glucosinolate composition of *Brassica* vegetables.

Keywords: glucosinolates, *Brassica* vegetables, Fourier Transform Infrared Spectroscopy

INTRODUCTION

Glucosinolates (β-thioglucoside-N-hydroxysulphates) are a very important plant secondary metabolites, specific to *Brassica* crops, like broccoli (*Brassica oleracea var. italica*), cauliflower (*Brassica oleracea* var. *botrytis*), kohlrabi (*Brassica oleracea* var. *gongylodes*) etc.

Glucosinolates (β-thioglucoside-N-hydroxysulphates) contains β-D-glucopyranose residues linked to a sulfur atom (i), or to a (Z)-N-hydroximinosulfate ester (ii) or a variable R group (iii) with different side-chain substituents which give their structural diversity (Fig.1 and 2).

Previous reports have shown that the ingestion of *Brassica* vegetables have a direct relationship with the decrease an incidence of different types of cancers (Cohen et al., 2000; Le Marchand et al., 1989; Van Poppel et al., 1999). The intact glucosinolates are biologically inactive, but, after the disruption of plant cell, they are rapidly hydrolysed by a myrosinase, a thioglucoside glycohydrolase, EC 3.2.3.1, to yield glucose and unstable aglycons that undergoes molecular rearrangement into di□erent breakdown products. For example, from glucoraphanin results sulforaphane, and from glucobrassicin, indole-3-carbinol. Recent research articles showed that these breakdown products induces phase-2 detoxification, boots

antioxidant status and protect the human body against cancer (Zanichelli et al., 2011, Abdul Razis et al., 2010; Abdul Razis et al., 2011, Wagner et al., 2009, Shapiro et al., 2001)

The quantification of total and individual glucosinolates from different samples is realized by a standard method (HPLC) is expensive and time-consuming, and need specialised personnel (Font et al., 2005). In contrast, using infrared spectroscopy, a fast analytical technique results in many advantages, e.g. short time of analysis, low cost/sample ratios and no use of hazardous chemicals. In many research papers, the infrared spectroscopy, and especially near-infrared spectroscopy (NIRS) has been used for qualitative and quantitative analysis of glucosinolates in seed of *Brassica* species (Biston et al., 1998; Velasco and Becker, 1998; Font et al., 2004), plant leaves (Font et al., 2005).

Font et al., (2005) tested the potential of near-infrared spectroscopy for screening the total glucosinolates content in the leaf rape (range between 1.06 – 49.18 μmol/g), where these compounds are present in significantly lower concentrations than those usually found in the seed.

Barbieri et al., 2008, found that the most abundant glucosinolates in broccoli were glucobrassicin and neoglucobrassicin, followed by glucoraphanin. Also, Hansen et al., (1995) found besides glucoraphanin, glucobrassicin, neoglucobrassicin, also glucoiberin, and 4-methoxyglucobrassicin as predominant glucosinolates in freshly "Marathon" broccoli sample. Tian et al., 2005, reported that besides glucoraphanin, the levels of glucobrassicin, glucoiberin, and neoglucobrassicin in broccoli is also consistent. In cauliflower, Tian et al., 2005, found that the sinigrin and glucoiberin are the major aliphatic GLS. From indole GLS, authors reported that glucobrassicin and 4-methoxyglucobrassicin were the major components, while neoglucobrassicin was present only in lower amount. Cabello-Hurtado et al., 2012, investigated the content of leaves and stalks from non-edible portion of cauliflower obtained from local agricultural production, and identified that the aliphatic GLS was the predominant comparative with indole GLS. Picchi et al., 2012, compared the GLS content from two genotypes of a green typology cauliflower. The glucobrassicin was the predominant component in all genotypes of cauliflower examined. There are few research manuscripts regarding to the glucosinolate content of kohlrabi (Yen and Wei, 1993; Carlson et al., 1987 and Sones et al., 1984).

Mid-IR spectroscopy, especially the ATR-FTIR (Fourier Transform Infrared Spectroscopy) was found to be a useful method to measure the release of isothiocyanates from plant glucosinolates in the soil, recognized in the region from 2174 to 2041 cm^{-1} specific to the –N=C=S functional group, a method successfully used for the detection of isothiocyanates in the soil (Li-Chan et al., 2010).

The objective of the present research was to analyze comparatively by HATR/FT-MIR Spectroscopy the fingerprint comparatively extracts of *Brassica* vegetables (broccoli, cauliflower, kohlrabi) cultivated in North-West of Romania and 9 pure glucosinolates (desulfo-sinigrin and sinigrin, desulfo-gluconapin, desulfo-progoitrin, desulfo-glucoiberin, desulfo-glucoraphanin, desulfo-glucotropeolin and glucotropeolin, desulfo-glucobrassicin).

MATERIALS AND METHODS

Plant material

The *Brassica* vegetables which were analyzed includes broccoli, cauliflower, and kohlrabi, all harvested in autumn (Octomber 2011) from ecological micro-farm with technologies that avoid the use of synthetic chemicals. After harvesting, the raw vegetable samples were rapidly frozen (at -20^0C), and then lyophilized using a freeze dryer Martin Christ Alpha 1-2 GmbH.

Pure glucosinolate standards

Purified standards of desulpho glucosinolates (sinigrin, gluconapin, progoitrin, glucoiberin, glucoraphanin, glucotropaeolin and glucobrassicin) and intact glucosinolates (sinigrin and glucotropaeolin) were kindly provided from dr. Renato Iori, Director of Research Industrial Crop Research Centre Agricultural Research Council, Italy.

Analysis of glucosinolates

Extraction of glucosinolates

The extraction of glucosinolates was made according to the EU official method (EEC Regulation N. 1864/90). Each raw sample was first ground and freeze dried to powder using a Martin Christ Alpha 1-2 LD equipment. The powder was stored at – 20^0C until extraction of glucosinolates. Aliquots of duplicate freeze-dried samples of 200 mg were extracted in 5 ml of aqueous methanol 70% for 5 min at 80^0C, and centrifuged at 5000 rpm for 20 minutes. The extraction was repeated in the same way on the solid residues. Supernatants were then combined and the total volume was measured. Each extract (1 ml) was analyzed twice by loading it onto a mini-column filled with 0.6 ml DEAE-Sephadex A-25 anion-exchange resin, preliminary conditioned with 25 mM acetate buffer pH 5.6. After washing with 3 ml of buffer, a volume of 200 μl purified sulphatase was loaded onto the mini-column which was left overnight at room temperature. The next day, the desulfo-glucosinolates were eluted with 3 ml of ultrapure water and finally analyzed by FT-MIR.

HATR/FT-MIR measurements

The Fourier Transform Mid-Infrared spectrum (FT-MIR) of each extract and pure glucosinolate standards were recorded from 4000 to 900 cm^{-1}. A number of 64 scans were accumulated for each spectrum using the Horizontal Attenuated Total Reflection (HATR) device, using a Shimadzu Prestige 2 FTIR spectrometer (with apodization Happ-Genzel). The spectral data were processed using the IR solution Software Overview (Shimadzu) and OriginR 7SR1 Software (OriginLab Corporation, Northampton, USA). The FT-MIR spectra were registered on the evaporated extracts, from both, desulpho – pure standards of glucosinolates and intact glucosinolates, mentioned above. The fingerprint of total glucosinolate extracts from three different *Brassica* vegetables (broccoli, cauliflower and kohlrabi) were determined by FTIR method, either using the intensity of the peak at 1058 cm^{-1} and 800 cm^{-1} or from the area of the regions 1100-1000 cm^{-1}, 1300-1160 cm^{-1}, 1300-1000 cm^{-1}.

A calibration curve with pure glucotropaeolin (range of concentrations 0.3 to 1.5 mg/ml) was obtained in parallel, for quantitative evaluations.

RESULTS AND DISCUSSION

HATR/FT-MIR fingerprint of pure standards

The integral HATR/FT-MIR spectra (3500 – 900 cm^{-1}) of nine glucosinolate pure standards (aliphatic, aromatic and indole glucosinolates) and three extracts of *Brassica* vegetables (broccoli, cauliflower and kohlrabi) were registered and the specific signals and wavenumbers were considered.

Table 1 includes the chemical structures of the main aliphatic, aromatic and indolic desulfo-glucosinolates which are found in *Brassica* vegetables after desuphatation with sulphatase: sinigrin (ds-SIN), gluconapin (ds-GNA), progoitrin (ds-PRO), glucoiberin (ds-GIB), glucoraphanin (ds-GRA) desulfo-glucotropaeolin (ds-GTL), desulfo-glucobrassicin (ds-GBS).

Three regions (marked A, B, C) were identified in the HATR/FT-MIR fingerprint domain (from 900 to 1680 cm^{-1}) (Fig.1-5): Region A (1100 -1000 cm^{-1}), corresponds to stretching vibrations of C-O bonds from monocarbohydrates (Vodnar et al.,2012; Zavoi et al., 2011; Chis et al., 2011), with the main signal at 1035 cm^{-1} for glucose and 1058 cm^{-1} for sucrose. Region B (1300-1100 cm^{-1}), corresponds to stretching vibrations of –SO-, while the region C (1550-1638 cm^{-1}) corresponds to indole heterocycle (with a maximum at 1590 cm^{-1}). The other regions, at more than 1700 cm^{-1} correspond to lipids, keto derivatives and OH-containing molecules, including water (>3276 cm^{-1}).

Identification of indole- and integral *vs* desulfated glucosinolates by HATR/FT-MIR fingerprints

Fig. 2 shows the comparative fingerprint of aromatic glucotropaeolin (GTL) before and after desulfation (ds-GTL) Major changes are visible in the regions A and B. The strong decrease of absorption in B region indicates the desulphuration.

Fig. 3 shows the comparative fingerprint of the aromatic ds-GTL and a mixture of ds-GTL with desulfated glucobrassicin (ds-GBS). Major changes are visible in the region C by a strong increase of absorption due to indole group.

Fig. 4 shows the comparative fingerprint of aliphatic sinigrin (SIN) before and after desulfation (ds-SIN). Major changes are visible in the regions A and B, as it was seen for GTL. The strong decrease of absorption in B region indicates the desulfation. The region C for this aliphatic glucosinolate is less complex than for GTL (aromatic derivative).

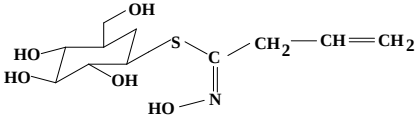
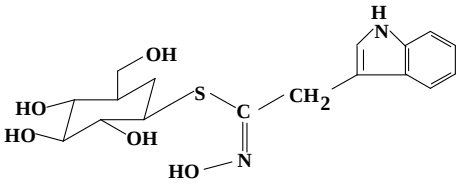
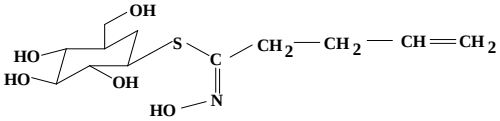
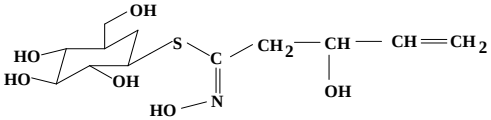
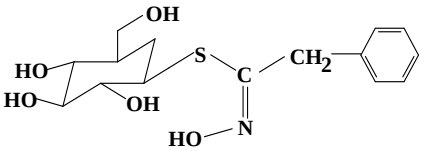
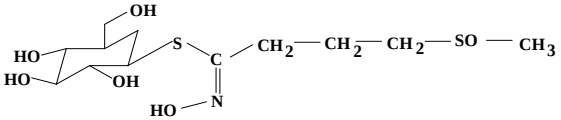
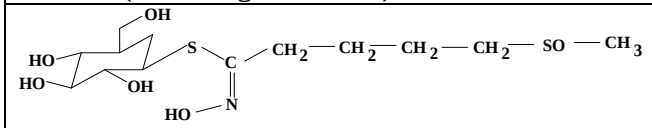
Calibration curve for glucosinolate quantification, using pure glucotropaeolin (GTL)

Fig. 1 represents the FT-MIR fingerprint of pure glucotropaeolin (GTL), used to make a calibration curve for total glucosinolates, using in the range of 0.3 to 1.5 mg/ml.

Based on FTIR registered bands of the following regions 1100-1000 cm^{-1}, 1300-1160 cm^{-1}, 1300-1000 cm^{-1}, the total glucosinolates of *Brassica* vegetables (broccoli, cauliflower and kohlrabi) were determined, by using considering the calibration curve with pure glucotropaeolin (range of concentrations 0.3 to 1.5 mg/ml). The calibration curve was determinated by linear regression, and the ecuations were: $y = 0.3357x – 0.1301$ ($R^2 = 0.9571$) for region 1100-1000 cm^{-1} (Region A), $y = 0,8954x – 0,2639$ ($R^2 = 0.9758$) for region B (1300 – 1160 cm^{-1}) and $y = 0,4421x – 0,3231$ ($R^2 = 0.9659$) for region 1300-1000 cm^{-1}, where x is area of the regions, and y is the concentration of glucosinolates (mg/ml). Also, the calibration curve was determined by recorded the signal intensity of glucosinolate at 1058 cm^{-1} and 800 cm^{-1}.

Table 1

The chemical structures of five aliphatic desulfo-glucosinolates which are found in *Brassica* vegetables: sinigrin (ds-SIN), gluconapin (ds-GNA), progoitrin (ds-PRO), glucoiberin (ds-GIB), glucoraphanin (ds-GRA) (column 1), the aromatic desulfo-glucotropaeolin (ds-GTL)(column 2) and indole desulfo-glucobrassicin (ds-GBS)(column 3) .

Aliphatic	**Indole or aromatic**
 ds-SIN (desulfo-sinigrin)	**INDOLE**  ds-GBS (desulfo-glucobrassicin)
 ds-GNA (desulfo-gluconapin)	
 ds-PRO (desulfo-progoitrin)	**AROMATIC**  ds-GTL (desulfo-glucotropaeolin)
 ds-GIB (desulfo-glucoiberin)	
 ds-GRA (desulfo-glucoraphanin)	

Comparative fingerprints of vegetable extracts after desulphatation of glucosinolates

Fig. 5 presents the specific fingerprint of the three vegetable (broccoli, cauliflower and kohlrabi) extracts after desulfation. It is obvious the bands disappearance from B region. The complexity of C region may indicate the presence of aromatic glucosinolates. Major changes are visible in the regions A and B, as it was seen for GTL. The strong decrease of absorption in B region indicates the desulfation. The region C for this aliphatic glucosinolate is less complex than for GTL (aromatic derivative).

Quantitative evaluation of total glucosinolates based on HATR/FT-MIR registration

Based on the calibration curve, the total glucosinolates of *Brassica* vegetables were expressed in mg glucotropaeolin eq. /100 g fresh weight, to allow for comparison with the database develop by McNaughton and Marks (2003). Our results, expressed as mg glucosinolates/ g fresh weight are shown in Table 2.

Table 2

The total glucosinolate of Brassica vegetables (mg glucotropaeolin eq. /100 g fresh weight) as calculated in relation to the the glucotropaeolin calibration curve

Peak area for a region or wavenumber (cm^{-1})	Broccoli	Kohlrabi	Cauliflower	Ratios B/K/C
1300-1160	**133.98**	110.94	73.29	1.82/1.51/1
1300-1000	**277.66**	270.72	182.41	1.52/1.48/1
750-900	**158.20**	123.44	71.92	**2.19/1.71/1**
1058	**62.10**	59.40	52.58	1.18/1.12/1
800	**74.10**	53.72	26.18	**2.83/2.05/1**

From all data it is obvious that broccoli is the richest source of glucosinolates, around two times more rich than cauliflower and around 1.2 times more rich than kohlrabi.

From the database (McNaughton and Marks, 2003) compiled from numerous research articles, the content of total glucosinolates of *Brassica* vegetables are situated between: 19.3-127.5 mg/100 g fresh weight for broccoli, 19.7-109.3 mg/100 g fresh weight for kohlrabi and 11.7-78.6 mg/100 g fresh weight for cauliflower. These data are in agreement with our data and ranking among the three species.

Regarding the most relevant absorption IR band to consider for the FT-MIR quantitative evaluation of glucosinolates, Yang et al., 1988, proposed for rapeseed meal to consider the absorption band at 800 cm^{-1}, absorbtion where the oil and meal protein do not absorb, and do not interfere. Considering our data, we can assume that either the signal area at 800 cm^{-1} or the absorption area of the region 750-900 cm^{-1} are apropriate to determine the content of glucosinolates in these extracts, as shown in Table 2.

Meanwhile, we consider that the quantification of glucosinolates considering the carbohydrate region (1300-1000 cm^{-1}) is over-estimated.

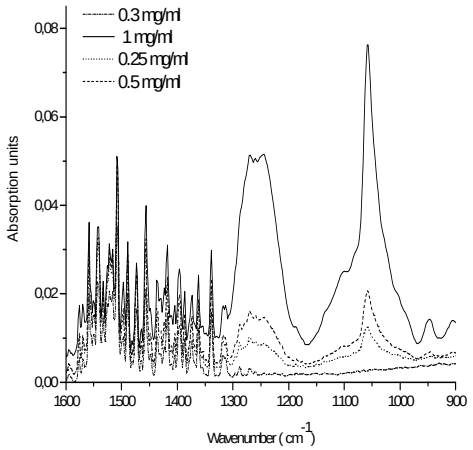


Fig. 1. FT-MIR fingerprint of pure glucotropaeolin (GTL), used to make a calibration curve for total glucosinolates, using in the range of 0.3 to 1.5 mg/ml.

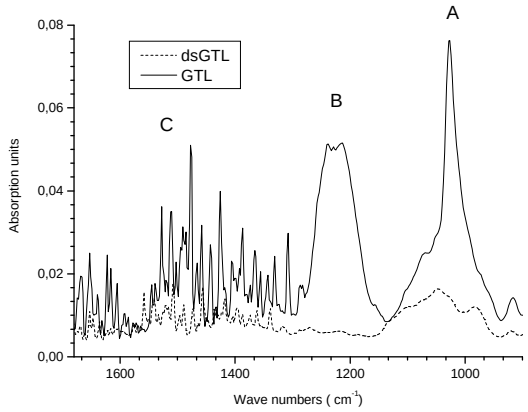


Fig. 2. Comparative fingerprint of aromatic glucotropaeolin (GTL) before and after desulfation (ds-GTL) Major changes are visible in the regions A and B. The strong decrease of absorption in B region indicates the desulphurization.

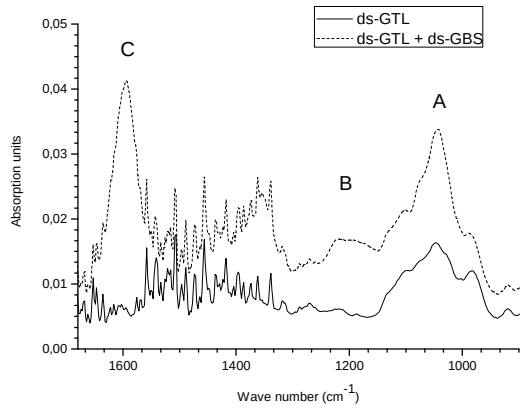


Fig. 3. Comparative fingerprint of the aromatic ds-GTL and a mixture of ds-GTL with desulphated glucobrassicin (ds-GBS). Major changes are visible in the region C by a strong increase of absorption due to indole group.

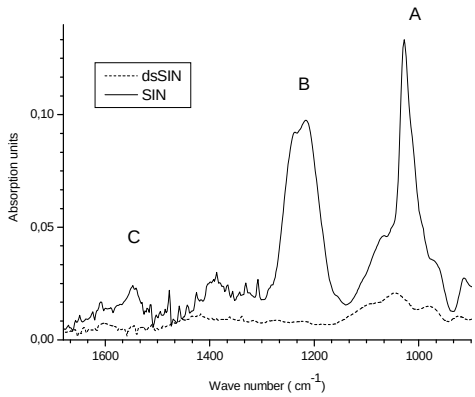


Fig. 4. Comparative fingerprint of aliphatic sinigrin (SIN) before and after desulfation (ds-SIN) Major changes are visible in the regions A and B, as it was seen for GTL. The strong decrease of absorption in B region indicates the desulfation. The region C for this aliphatic glucosinolate is less complex than for GTL (aromatic derivative).

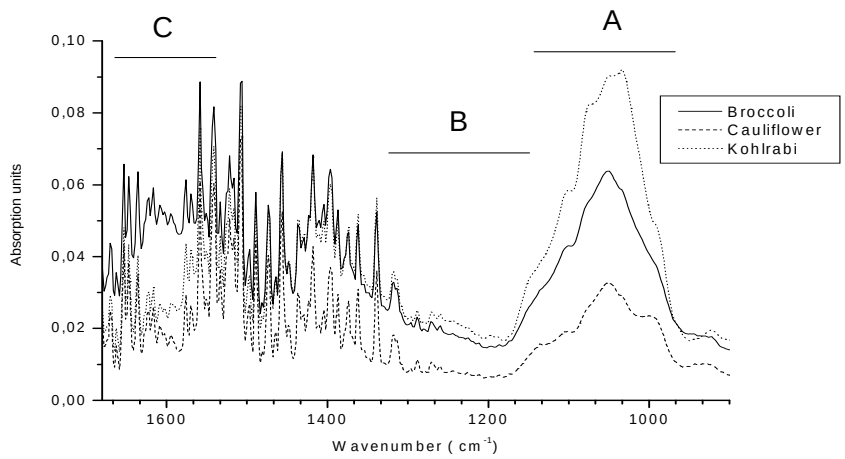


Fig. 5. Comparative fingerprint of vegetable extracts after desulfation. Details about the procedure used, in Materials and methods. It is obvious the disappearance of peaks from B region. The complexity of C region may indicate the presence of aromatic glucosinolates. Major changes are visible in the regions A and B, as it was seen for GTL. The strong decrease of absorption in B region indicates the desulfation. The region C for this our samples is less complex than for GTL (aromatic derivative).

Generally, according to literature reports, the analysis of glucosinolates in *Brassica* vegetables are based on their enzymatic O-desulfation, followed by the HPLC separation. In this paper, we recorded the HATR/FT-MIR spectra for both intact glucosinolates (some pure molecules) and desulfo-glucosinolates which are found in these vegetables. In the spectra are identified constantly the region A, specific to carbohydrate moiety, as well the disappearance of the SO specific peak when desulfation is finalized. Region C corresponds mainly to indole-containing glucosinolates and the complexity of this region depends also on the dominance of aliphatic or aromatic derivatives (see Fig. 2 and 4).

By the HATR/FT-MIR spectroscopy can be measured, in a rapid and non-destructive manner the extracts before and after enzymatic hydrolysis, evaluating if the hydrolysis is complete or not, information which is necessary for further detailed HPLC analysis.

When the content of glucosinolates was determined by HPLC in the same extracts (data not shown) we observed similar rankings, the glucosinolates content being the highest in broccoli, followed by kohlrabi and cauliflower, at similar levels.

CONCLUSION

The results presented in this paper showed that HATR/FT-MIR spectroscopy is an adequate technique to fingerprint comparatively the glucosinolates, as raw extracts and desulfated ones.

Also, this technique is easier to screen the vegetable extracts, the enzyme-assisted yield of desufation. If HPLC remains the “golden standard” method for identification, FT-MIR, by correct calibrations and validation, can be an excellent alternative to investigate in short time and more cheap, many extracts in short time. As well, HATR/FT-MIR can be used as a fast method for the quantification of total glucosinolates considering the IR absorption at 800 cm^{-1}or the region area of 750-900 cm^{-1}, is the most suitable. However, based on these data and comparative evaluations by HPLC, we consider the HATR/FT-MIR method to be an

appropriate method, non-destructive, cheap and fast to fingerprint, to predict and to quantify the glucosinolate composition of *Brassica* vegetables.

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