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Sample Preparation Optimization for the Analysis of Gliadins in Food by Matrix-assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry

Emilio Camafeita,[1] Patricia Alfonso,[2] Boris Acevedo[1] and Enrique Méndez[1,2]
[1]Unidad de Análisis Estructural de Proteínas, Centro Nacional de Biotecnología, Cantoblanco, 28049 Madrid, Spain
[2]Servicio de Endocrinología, Hospital Ramón y Cajal, 28034 Madrid, Spain

Analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI/TOF-MS) of wheat gliadin extracts from distinct types of wheat varieties displays a similar characteristic gliadin mass pattern within the 30–40 kDa range. Direct observation of this pattern in food samples was used to identify gluten gliadins in food rapidly and easily. A modified sample preparation for gliadin analysis by MALDI/TOF-MS has improved sensitivity of gliadin detection by a factor of more than 50. This procedure allows the direct detection of complex mixtures of gluten gliadins in food sample extracts down to a concentration of 0.01 mg ml^{-1}, i.e. down to levels which are toxic for coeliac patients. This highly sensitive improved method has been also exemplified by the facile detection of individual proteins at low concentrations (100–400 pmol ml^{-1}). © **1997 by John Wiley & Sons, Ltd.**

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INTRODUCTION

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI/TOF-MS) has become a powerful analytical method for biological mass spectrometry.[1,2] Recently, this technique has been successfully used for the direct analysis of unfractionated alcohol-soluble prolamin fractions of gluten from wheat, barley, rye and oats, so-called gliadins, hordeins, secalins and avenins, respectively.[3,4] These cereal protein components, which are frequently present in common diet, are toxic to patients with Coeliac disease (CD) (gluten-sensitive enteropathy) and their ingestion provokes severe mucosal lesions in the small intestine in such patients.[5,6] The treatment of CD consists simply in the elimination of gluten from the diet of coeliac patients, who must consume only gluten-free foods. The gluten content of these products must be under 5–10 mg of gliadins per 100 g of food.[7,8] Conventionally, several immunological procedures, viz. enzyme-linked immunosorbent assay (ELISA),[9–15] immunoblotting[16] and, more recently, polymerase chain reaction (PCR),[17] have been used to analyse gluten in food. Nevertheless, none of these conventional methods is capable of yielding the gluten content in food with sufficient reliability to permit better dietary control.

We recently suggested a novel alternative method for the rapid and easy detection of gluten in food by the direct observation of the characteristic gliadin mass pattern in food samples using MALDI/TOF-MS.[3] We now report preliminary data on the optimization of the sample preparation method prior to MALDI/TOF-MS which allows the measurement of gliadin concentrations at toxic levels.

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EXPERIMENTAL

Materials

Wheat (Triticum durum L. cv. Senatore Capelli, Triticum durum L. cv. Araldun, Triticum aestivum L. cv. Minaret, Triticum aestivum L. cv. Stratos, Triticum aestivum L. cv. Ralle, Triticum aestivum L. cv. Taurus and Triticum spelta) seeds were used. The gliadin standard froma gluten assay kit (Cortecs Diagnostic, Deeside, UK)was used. Bovine serum albumin (BSA) and horse heart cytochrome c (CC) were purchased from Sigma (St Louis, MO, USA). Two commercially available gluten-free food samples (biscuit and pap) from batches contaminated with gliadins, probably during the elaboration process, were used.

Reagents

Acetonitrile, trifluoroacetic acid and 2-mercaptoethanol were obtained from Merck (Darmstadt, Germany), ethanol from Scharlau (Barcelona, Spain) and sinapinic (trans-3,5-dimethoxy-4-hydroxycinnamic) acid and octyl-β-D-glucopyranoside from Fluka (Buchs,

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Switzerland). Ultra-pure water from a Milli-Q purification system (Millipore, Bedford, MA, USA) was used in the preparation of all solutions.

Preparation of protein extracts

Wheat and food flours were extracted for 1.5 h in a shaker at room temperature using 40% (v/v) aqueous ethanol (1 g in 10 ml). Samples were centrifuged at 4700 g for 15 min and the supernatant was mass analysed.

ELISA gluten assay

The gluten assay kit (Cortecs Diagnostic, Deeside, UK) was employed following the manufacturer's instructions. This assay consists of a sandwich ELISA which uses an anti-ω-gliadin monoclonal antibody for the coating and the same antibody as a peroxidase conjugate.[18]

Sample preparation and mass spectrometry

A 2, 20, 40 or 80 µl volume of sample containing 0.2, 1.6, 2.7 and 4 mM octyl-β-D-glucopyranoside detergent was mixed with a 100 µl fixed volume of a saturated solution of sinapinic acid in 30% aqueous acetonitrile and 0.1% trifluoroacetic acid used as a matrix. A 1 µl volume of this mixture was then deposited on a stainless-steel probe tip and allowed to dry at room temperature for 5 min.

Samples were measured on a Bruker (Bremen, Germany) Reflex II MALDI/TOF mass spectrometer equipped with an ion source with visualization optics and a nitrogen laser (337 nm). Mass spectra were recorded in the linear positive mode at 30 kV acceleration voltage and 2 kV in the linear detector by accumulating 70 spectra of single laser shots under threshold irradiance. The equipment was externally calibrated with a mixture of BSA (66430 Da) and CC (12360 Da).

RESULTS AND DISCUSSION

MALDI/TOF analysis of different wheat cultivars

We recently reported that unfractionated wheat gliadins yield a characteristic protonated mass pattern by MALDI/TOF-MS. The results suggested that this pattern could be used for the identification of gliadins in food by simply observing the presence of these characteristic masses in food samples. The original study was performed with a single wheat cultivar. To demonstrate the general validity of this procedure, we have now investigated the occurrence of this mass signal pattern in a large number (30) of wheat cultivars.

Figure 1 shows MALDI/TOF spectra of the 40% ethanol gliadin extract from six of those wheat cultivars: Minaret, Spelta and Senatore Capelli (left) and Stratos, Ralle and Taurus (right). The spectra show that all cultivars yield a very similar characteristic protonated gliadin mass pattern with only minor mass differences in the two main groups of mass signals at around 31 and 33–35 kDa (see area selected by a box). These minor differences are probably due to polymorphism of α- and γ-type gliadins, with respect to both charge and size, amongst different cultivars.[19–22] This behaviour was also observed for the remaining 24 wheat cultivars analysed (data not shown). A characteristic gliadin mass pattern is obtained regardless of the particular wheat cultivar and this is therefore the mass pattern expected in gliadin-containing food samples. Its appearance could, in principle, be employed to identify gliadins in gluten-free foods commonly consumed by coeliac

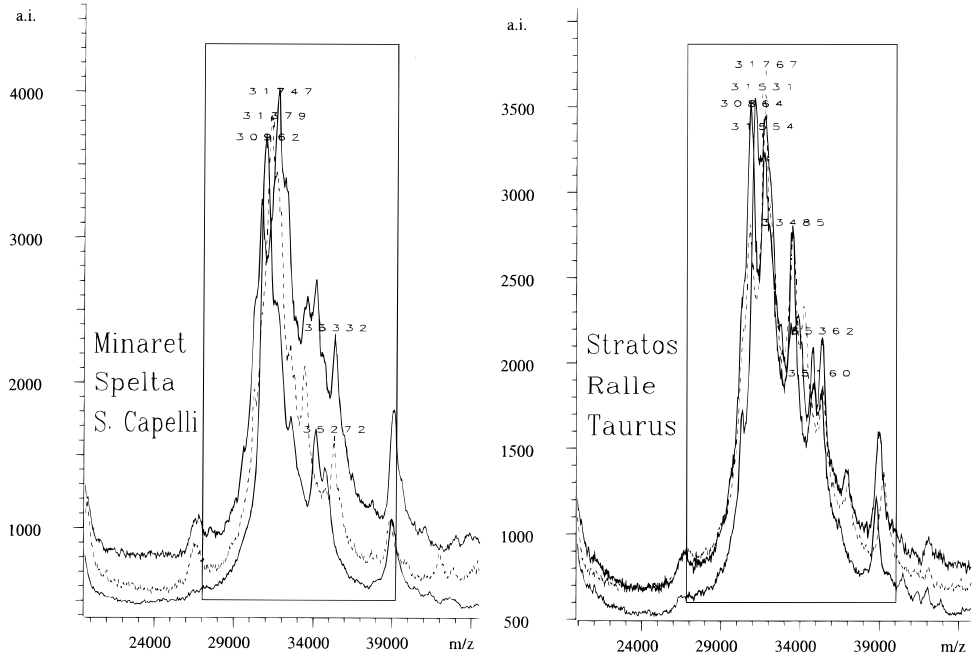

Figure 1. MALDI/TOF mass spectra of the gliadin extracts (100 mg ml^{-1}) from six different wheat cultivars. A selected gliadin mass range from 27–40 kDa is indicated by a box.

patients, provided that the detection sensitivity of MALDI/TOF is sufficient to determine quantities of gliadins in food at or around the toxicity limit.

Detection sensitivity of gliadins by MALDI/TOF-MS

The mass analyses presented in Fig. 1 were carried out using 2 μl of a 100 mg ml^{-1} 40% ethanol extract from the corresponding flour. In order to determine the lowest concentration which permits the visualization of the characteristic gliadin mass pattern, dilutions from 10 down to 0.5 mg ml^{-1} of the 40% ethanol extract from the wheat cultivar Araldun were mass-analysed. Figure 2 (left) shows that although both the intensity and resolution of gliadin mass signals decrease on lowering the concentration, the gliadin mass pattern can be still recognized at 2 mg ml^{-1}. However, this concentration is insufficient in comparison with the gliadins' toxicity limit in gluten-free foods. Accordingly, MALDI/TOF-MS cannot be used as a tool to detect gliadins in gluten-free foods by this experimental procedure.

In order to optimize conditions that permit the visualization of the gliadin mass pattern at much lower gliadin concentrations, the current sample preparation method using 2 μl of ethanol extract was modified by adding 20, 40 and 80 μl of ethanol extract to a 100 μl fixed volume of matrix. Figure 3 (left) shows the intensity of the gliadin mass pattern as a function of sample volume added. To measure the intensity of this mass pattern, the absolute intensity of the most prominent α-gliadin mass signal at around 30 kDa (Fig. 2 (left)) was chosen. This result shows that, while intensity is very low when using 2 μl of the 0.5 mg ml^{-1} ethanol

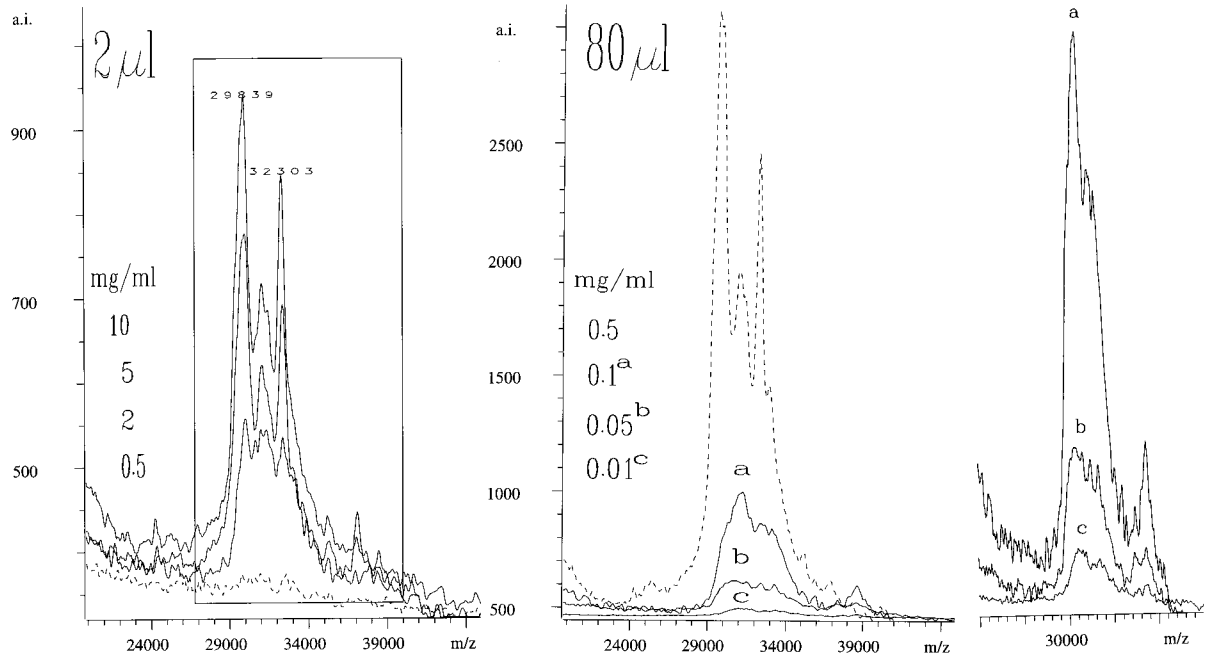

Figure 2. Comparison of MALDI/TOF mass spectra of the extract from *Triticum durum Araldun* using sample volumes of 2 μl (left) and 80 μl (centre) at decreasing extract concentrations as indicated (top to bottom). Spectra corresponding to 0.5 mg ml^{-1} are displayed as a dashed line for comparison. A more detailed, enlarged view of spectra a, b and c is included (right).

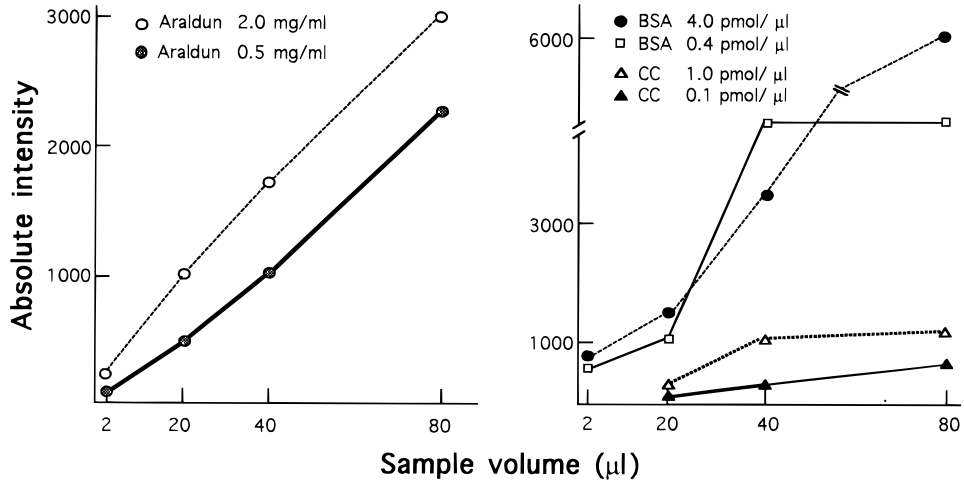

Figure 3. Mass signal absolute intensity *vs*. sample volume. For the gliadin extracts (left) the absolute intensity of the most intense mass signal at ~ 31 kDa was monitored and for BSA and CC the corresponding singly charged mass signal (right). For comparison, different concentrations were employed as indicated.

extract (Fig. 2 (left), see dashed spectrum), it increases as the added volume is increased. The same linear response was found when using this ethanol extract at a higher concentration of 2 mg ml^{-1} (Fig. 3 (left)).

The utility of increasing sample volume is depicted in Fig. 2. While the gliadin mass pattern was nearly undetectable when using a 2 μl volume of 0.5 mg ml^{-1} extract (Fig. 2 (left), see dashed spectrum), it became very intense when using 80 μl (Fig. 2 (centre and right), see dashed spectrum). This improvement has permitted to increase the previous detection sensitivity[3] by a factor of 50–100 and visualization of the gliadin mass pattern down to a concentration of 0.01 mg ml^{-1} (Fig. 2 (centre and right)). This represents an acceptable sensitivity taking into account that gliadin extracts are very complex mixtures containing up to 50 gliadin components.[19–21] Moreover, the above limit concentration calculated by weighting is an overestimation given that proteins constitute only a fraction of the extract components as determined by amino acid analysis.

This improvement, based on the use of increasing volumes, is demonstrated to be useful as a systematic procedure to increase the detection sensitivity when mass analysing proteins at low concentrations. Figure 3 (right) shows a rise in absolute intensity of BSA and CC singly charged signals as the volume added increases (spectra not shown), which allowed the detection of BSA and CC at concentrations of 0.4 and 0.1 pmol/μl, respectively.

Determination of the detection limit with a gliadin standard

To estimate the detection sensitivity for gliadins by MALDI/TOF, dilutions of a gliadin standard ranging from 0.1 down to 0.005 mg ml^{-1} were mass analysed under the improved conditions above. No mass spectrum was obtained below 0.01 mg ml^{-1}.

Figure 4 displays the absolute intensity of the most intense α-gliadin mass signal at ~ 30 kDa as a function of the standard concentration. Accordingly, this procedure allows the visualization of the gliadin mass pattern (spectra not shown) down to a concentration of 0.01 mg ml^{-1}. This value corresponds to 10 mg of gliadins per 100 g of food, the toxic threshold in gluten-free foods.

MALDI/TOF analysis of gliadin-contaminated gluten-free foods

The efficiency of this improved method was demonstrated by analysing two gliadin-contaminated gluten-free foods. Figure 5 shows that although both samples (biscuit (left) and pap (right)) are highly contaminated (88 and 144 mg per 100 g of food as determined by ELISA), no gliadin mass signals are detected when using 2 μl (dashed spectra) while an intense, well defined

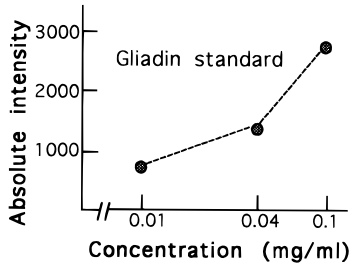

Figure 4. Mass signal absolute intensity *vs.* sample concentration for the gliadin standard. The absolute intensity of the most intense mass signal at ~31 kDa was monitored.

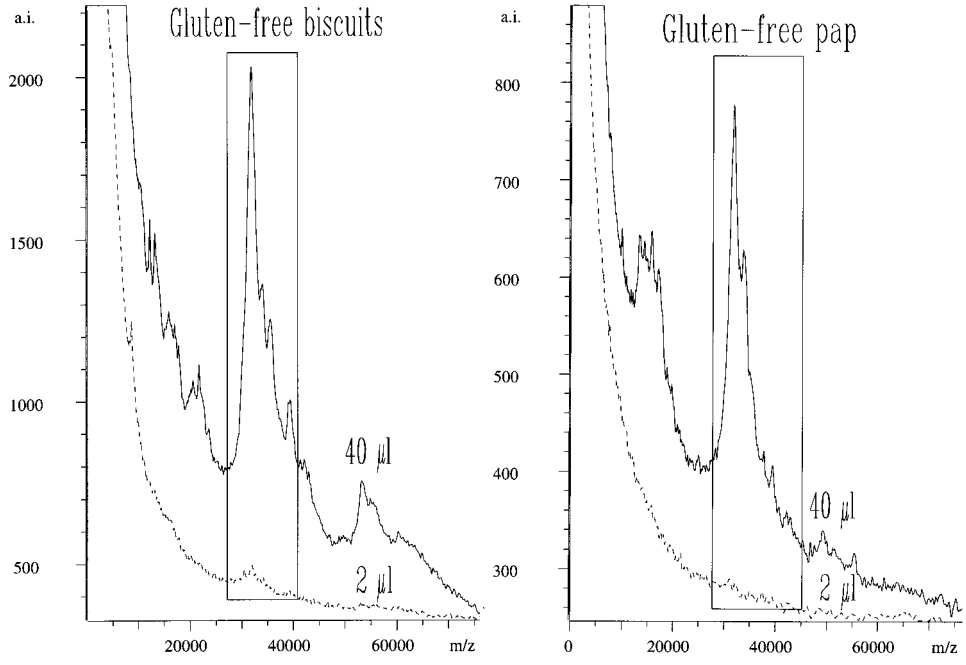

Figure 5. MALDI/TOF mass spectra of the gliadin extracts from two gliadin-contaminated gluten-free foods, biscuit (left) and pap (right), using 2 and 40 μl of sample. The characteristic gliadin mass range is indicated by a box and the gluten content as determined by ELISA is displayed.

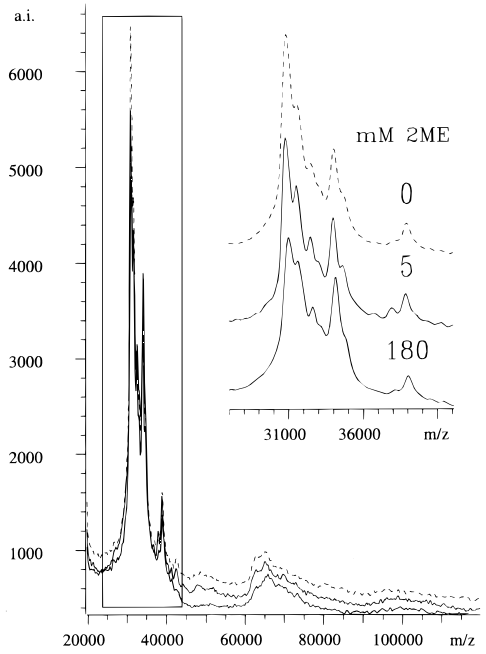

Figure 6. MALDI/TOF mass spectra of the gliadin extract from *Triticum durum Senatore Capelli* before and after reduction with 5 and 180 mM 2-mercaptoethanoln(top to bottom). The characteristic gliadin mass range has been expanded in the inset for a better comparison.

mass pattern shows when employing a higher sample volume of 40 µl. Accordingly, this procedure has permitted the clear detection of gliadins in food which otherwise would remain undetected by MALDI/TOF.

Analysis of wheat gliadins by MALDI/TOF-MS under reducing conditions

To date, one of the problems remaining to be resolved for the analysis of gluten in food is to optimize a quantitative gliadin extraction procedure. Under standard ethanol extraction conditions, an unknown proportion of the gliadins link by disulphide bonds to glutenins and are not extracted.[14,23]. Extraction of these gliadins involves the use of reducing agents such as 2-mercaptoethanol or dithiothreitol. However, these reagents are not compatible with ELISA, especially at low dilutions, as they damage the antibody. To investigate the effect of these reducing agents on the identification of wheat gliadins by MALDI/TOF-MS, an ethanol gliadin extract was incubated with different concentrations of 2-mercaptoethanol (5, 20, 50, 100 and 180 mM). Figure 6 shows that no significant changes are observed in the gliadin mass pattern, even at high concentrations of 2-mercaptoethanol (180 mM). Hence, if a complete gliadin extraction requires the use of reducing agents this will not be a handicap for analysis by MALDI/TOF-MS, unlike the case with ELISA systems.

CONCLUSIONS

The results show that MALDI/TOF-MS is an appropriate tool for the identification of wheat gliadins in food by observing the characteristic protonated gliadin mass pattern in food samples. This mass pattern has been confirmed not to depend greatly on the particular wheat cultivar.

The optimized MALDI/TOF procedure based on the use of an increased sample volume has permitted (i) the detection of gliadins down to 0.01 mg ml^{-1}, the toxicity limit for coeliac patients (the validity of this procedure has also been confirmed for other proteins at low concentrations, as demonstrated for BSA and CC) and (ii) the detection of gliadins in gluten-free samples which remained undetected by the previously reported procedure. Furthermore, reducing agents do not affect the gliadin mass pattern obtained by MALDI/TOF. These features support the use of MALDI/TOF as a tool to determine gluten gliadins in food.

This preliminary study constitutes a preliminary effort to establish the MALDI/TOF-MS technique as a routine system for the detection of gluten in food as an alternative to conventional ELISA. The success of this task will depend on the solution of additional experimental challenges such as (i) increased detection sensitivity down to 0.1–1 mg of gliadins per 100 g of food, (ii) whether a linear response can be obtained to determine if the technique is suitable for semi-or quantitative analysis, (iii) the consistency between MALDI/TOF data and ELISA values, (iv) the establishment of an efficient, quantitative gliadin extraction procedure and (v) the use of a cereal protein as an internal standard to measure concentrations of wheat proteins. All these aspects are currently under study.

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