Ion-Exchange Chromatographic Method for the Determination of the Free Amino Acid Composition of Cheese and Other Dairy Products: an Inter-Laboratory Validation Study

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Abstract Although free amino acids (FAAs) represent a significant component of ripened cheeses and can provide useful information for their characterization, no inter-laboratory validated analytical method exists which allows a reliable comparison of data obtained by different laboratories and the adoption of quality control schemes based on FAA pattern. The objective of the present work was to test the effectiveness of an analytical protocol for the determination of the FAA composition of cheese and to verify the adequateness of this type of analysis for quality control procedures of Grana Padano PDO cheese as well as for research purposes. After an initial test to compare performances of ion-exchange chromatography (IEC) and HPLC techniques, an inter-laboratory collaborative study (seven laboratories, four samples) was organized to validate an IEC method with post-column ninhydrin derivatization and using L-norleucine as an internal standard. Determined amounts of individual FAA ranged from 8 to over 1380 mg/100 g cheese, with relative standard deviation of repeatability (RSD\(_r\)) ranging from 0.5 to 4.6%, and relative standard deviation of reproducibility (RSD\(_R\)) ranging from 1.3 to 9.9% for FAA concentrations over 100 mg/100 g. For lower concentrations, RSD\(_r\) and RSD\(_R\) were about thrice as high. On the basis of the results of this investigation, at present, the validated method is adopted as the official method for the determination of FAA patterns in the quality control of Grana Padano PDO cheese.

Keywords Free amino acids · Cheese · Ion-exchange chromatography · Inter-laboratory study · Precision · Method validation

Introduction Although free amino acids (FAAs) are usually considered minor cheese constituents, they have been shown to contribute to sensory properties (Toelstede et al. 2009; Zhao et al. 2016), nutritional characteristics (Bottesini et al. 2013), and physiological functions (San Gabriel and Uneyama 2013) of several cheese varieties. During cheese ripening, protein is progressively degraded by a number of proteolytic enzymes including (1) chymosin, (2) indigenous milk proteases, and (3) proteases and peptidases from both starter (LAB) and non-starter lactic acid bacteria (NSLAB), mainly released after cell lysis (Borsting et al. 2012; Gatti et al. 2014). According to the manufacturing process and ripening period, up to 20–25% of the cheese protein may be split into FAAs, which can represent over 50% of the soluble N fraction (Sousa et al. 2001; Pellegrino et al. 2013). In long ripened cheeses, FAA patterns have been investigated as a possible tool for characterizing the ripening process. Whereas some FAAs, such as lysine, alanine, glycine, and serine, are rather stable and mostly accumulate over time (Resmini et al. 1985; Frau et al. 1997), others undergo degradation phenomena through specific metabolic pathways of LAB (Liu et al. 2003; Ardö 2006). Furthermore, some non-protein amino acids (AAs), principally ornithine, citrulline, and γ-aminobutyric acid, are formed that may represent characteristic traits of certain cheeses (Nomura et al.
Several Protected Designation of Origin (PDO) cheeses, such as Parmigiano-Reggiano (Resmini et al. 1985), Mahon (Polo et al. 1985), Grana Padano (Resmini et al. 1993; Cattaneo et al. 2008; Masotti et al. 2010), Emmentaler (Krause et al. 1997), Montasio (Innocente 1997), Gruyère and Sbrinz (Bütikofer and Fuchs 1997), and Manchego (Poveda et al. 2004), have been shown to have characteristic FAA patterns. The common rationale behind this fact is that all these cheeses (1) are made from raw milk produced in a restricted geographical area, (2) following a well-defined traditional manufacturing process, and (3) using a natural whey culture daily prepared from the previous cheesemaking. These provisions are detailed in the product specification for PDO protection (European Council 2012) and guarantee that the same relevant microbial species (LAB and NSLAB) are constantly transferred from milk into the cheese (Gatti et al. 2014). As a result, for each cheese type, the proteolytic pathways occurring during ripening are repeatable and hence the resulting FAA pattern as well is repeatable and characteristic. Masotti et al. (2010) determined the FAA pattern of 150 samples of Grana Padano PDO cheese demonstrating that, on the basis of the relative amount of a selected group of FAAs, it is possible to recognize the authentic PDO cheeses from imitation cheeses with high statistical reliability ($p < 0.01$). Due to the power of this analytical approach as a tool for recognizing the authentic PDO cheeses, the respective FAA patterns have been introduced into the product specification among the characteristic traits for both Grana Padano (European Commission 2011a) and Parmigiano-Reggiano (European Commission 2011b).

Several analytical techniques have been proposed for AA determination, principally based on either reversed-phase (RP) HPLC or on ion-exchange chromatography (IEC). Bütikofer and Ardö (1999) demonstrated that the latter technique gives more reliable results in cheese analysis, despite the disadvantage of requiring a dedicated equipment. Since the first time that Moore et al. (1958) proposed the determination of AA by IEC coupled with post-column derivatization with ninhydrin, fully automated instruments have been developed, making this analysis feasible on a routine basis and applicable in research studies in many fields. Despite this, very few methods have been validated at inter-laboratory level (AOAC 1994; European Commission 2009) and, to the author’s knowledge, no one dealing with food products. Inter-laboratory validated methods allow to compare figures from different studies, provide reliable data to set up quality control schemes, and represent a useful tool for laboratories to assess their own performances.

This paper reports the work conducted to fully validate a method for the determination of the FAA content in cheese that was previously in-house validated and proved to be suitable for cheese characterization. This method includes both the extraction procedure and the chromatographic separation. Several laboratories have been involved, in order to validate it according to the internationally accepted protocols. A total of 21 FAAs were considered, including non-protein amino acids that proved to be present in ripened cheese. A preliminary pilot test was conducted to assess whether HPLC and IEC could give comparable results, and thus, both the techniques could be considered in the validation study. Finally, the suitability of the validated method to control authenticity of Grana Padano PDO cheese was tested using a simple statistical model that we developed in previous studies.

**Materials and Methods**

**Chemicals**

All reagents, employed for both the FAA extraction and separation, were of analytical grade or higher. $\alpha$-amino acids were from Sigma-Aldrich (Milan, Italy), except isoleucine from Merck KGaA (Darmstadt, Germany).

**Amino Acid Standard Solutions**

For the pilot test, a set of ready-to-use amino acid standards at five different concentrations was prepared at the Department of Food, Environmental and Nutritional Sciences (DeFENS) of the State University of Milan (the coordinating laboratory) and shipped to all participants.

For the collaborative study, a stock solution was prepared at the coordinating laboratory by weighing into a 200-mL volumetric flask:

- 30 mg of $\gamma$-aminobutyric acid (Gaba), citrulline (Cit), glycine (Gly), and glutamine (Gln);
- 40 mg of alanine (Ala), arginine (Arg), asparagine (Asn), methionine (Met), ornithine (Orn), threonine (Thr), and tyrosine (Tyr);
- 60 mg of aspartic acid (Asp), histidine (His), isoleucine (Ile), phenylalanine (Phe), and serine (Ser);
- 80 mg of leucine (Leu), proline (Pro), and valine (Val);
- 90 mg of glutamic acid (Glu) and lysine (Lys)

and making up to the mark with 0.2 N tri-sodium citrate buffer (SCB) at pH 2.2. An internal standard solution (60 mg L-norleucine in 100 mL SCB) was prepared as well. At the participating laboratories, aliquots of 0.5, 1, 2, and 5 mL of the stock AA standard solution were then transferred into 100-mL volumetric flasks, added with 2 mL of the internal standard solution and made up to the mark with SCB to prepare working solutions at four different concentrations.
Cheese Samples

Four samples of Grana Padano PDO cheese (samples A–D) of known age (9, 12, 18, and 22 months) were used for the pilot test. For the validation study, four samples of Grana Padano PDO cheese (samples 1–4) of known age (6, 12, 16, and 20 months) were used. Cheese samples were kindly provided by the Consorzio di Tutela del Formaggio Grana Padano.

All cheeses were sampled according to ISO Standard 707:2008 (ISO 2008), finely ground and thoroughly mixed, then divided into 10-g portions, sealed under vacuum in small plastic bags, and kept frozen (−32 °C) until shipping. All samples were assigned a serial number (blind samples) before being sent to participants. Samples for the validation study were tested for homogeneity and stability according to the ISO Standard 13528 (ISO 2015).

Organization of the Pilot Test

Fifteen experienced laboratories participated in a tentative pilot test, seven using IEC with ninhydrin post-column derivatization and eight using reversed-phase HPLC with o-phthalaldehyde (OPA) pre-column derivatization. Laboratories were supplied with a set of five AA standards for calibration and were asked to analyze each of twelve cheeses (three blind replicates of four different cheeses), sticking to the protocol for the FAA extraction procedure and using their own chromatographic conditions without any restrictions. Laboratories using HPLC generally adopted a Hypersil ODS column 250 × 4 mm, a 24-min stepwise linear two-solvent gradient (solvent A, 30 mmol/L NaOAc pH 7.20 + 0.25% tetrahydrofuran + 0.1 mol/L titriplex III; solvent B: 100 mmol/L NaOAc pH 7.20 + 80% acetonitrile + 0.1 mol/L titriplex III), flow rate 1.00 mL/min, column temperature 42 °C, and fluorescence detection (Ex: 340 nm and Em: 455 nm), as reported by Büttikofer and Ardö (1999).

Protocol for the Determination of Free Amino Acids by Ion-Exchange Chromatography

Six different elution buffers are employed; buffer composition is indicated in Table 1. All buffers, except buffer 6, are added with 0.1 mL/L of pentachlorophenol (500 mg/100 mL ethanol) as a preservative; buffers 1, 2, and 3 are added with 8.0 mL/L of a 25% (v/v) thiourea water solution and buffers 1 and 2 with 15 mL/L of isopropyl alcohol.
FAAs are separated using the gradient of pH, ionic strength, and temperature reported in Table 2; ninhydrin flow rate is 20.0 mL/h. Injection volume is 100 μL.

**Statistical Analysis**

Results obtained in the pilot study were evaluated by calculating mean values and relative standard deviations (RSDs) for every single FAA determined in all four samples both by IEC and by HPLC. Significant differences between data obtained by the two techniques were detected by Student’s t test. Statistical evaluation of the data of the collaborative study and calculation of the precision figures (means, standard deviation and relative standard deviation of repeatability and of reproducibility, repeatability, and reproducibility limits) were carried out according to ISO Standard 5725 (ISO 2004). Detection of outliers was performed by Cochran’s C test for abnormal variances and Grubbs’ test for abnormal mean values.

**Results and Discussion**

**Pilot Test**

The mean values of the total content of the 17 FAA determined in the four test samples were comparable between the two techniques, but variability was much higher for HPLC data (Fig. 1). Overall, contents of individual FAAs approximately ranged from 50 mg/100 g cheese (glutamine and

| Step | Duration | Temperature (°C) | Buffer | Flow rate (mL/h) | Ninhydrin |
|------|----------|------------------|--------|-----------------|-----------|
| 1    | 01:00    | 32               | 1      | 20              | On        |
| 2    | 01:00    | 32               | 1      | 20              | On        |
| 3    | 01:00    | 32               | 1      | 20              | On        |
| 4    | 05:30    | 32               | 1      | 20              | On        |
| 5    | 43:00    | 32               | 2      | 20              | On        |
| 6    | 17:00    | 40               | 3      | 20              | On        |
| 7    | 10:00    | 64               | 3      | 20              | On        |
| 8    | 34:00    | 64               | 4      | 20              | On        |
| 9    | 50:00    | 76               | 5      | 20              | On        |
| 10   | 06:00    | 76               | 6      | 20              | On        |
| 11   | 10:00    | 32               | 1      | 20              | On        |
| 12   | 01:00    | 32               | 1      | Off             | Off       |
| 13   | 25:00    | 32               | 1      | 25              | Off       |
| 14   | 10:00    | 32               | 1      | 20              | On        |

Fig. 1 Mean values and ranges of total free amino acid (FAA) content (mg/100 g cheese \(10^{-3}\)) in four cheese samples analyzed by IEC and HPLC.
|      | Sample A IEC | Sample A HPLC | Sample B IEC | Sample B HPLC | Sample C IEC | Sample C HPLC | Sample D IEC | Sample D HPLC |
|------|--------------|---------------|--------------|---------------|--------------|---------------|--------------|---------------|
| Asp  | 103          | 89            | 182          | 158           | 340          | 311           | 351          | 338           |
| RSD %| 37           | 37            | 23           | 38            | 16           | 36            | 23           | 34            |
|      | p < 0.05     |               |              |               |              |               |              |               |
| Thr  | 127          | 163           | 217          | 223           | 206          | 401           | 190          | 343           |
| RSD %| 20           | 48            | 18           | 37            | 17           | 72            | 18           | 66            |
|      | p < 0.05     |               |              |               |              |               |              |               |
| Ser  | 165          | 200           | 308          | 272           | 427          | 510           | 480          | 592           |
| RSD %| 24           | 41            | 17           | 37            | 17           | 33            | 20           | 31            |
|      | p < 0.05     |               |              |               |              |               |              |               |
| Asn  | 140          | 235           | 201          | 252           | 189          | 336           | 155          | 284           |
| RSD %| 30           | 33            | 30           | 35            | 28           | 38            | 34           | 35            |
|      | p < 0.05     |               |              |               |              |               |              |               |
| Glu  | 690          | 711           | 1000         | 803           | 1474         | 1487          | 1442         | 1508          |
| RSD %| 19           | 32            | 19           | 35            | 25           | 36            | 27           | 36            |
|      | p < 0.05     |               |              |               |              |               |              |               |
| Gln  | 171          | 219           | 115          | 131           | 48           | 94            | 132          | 65            |
| RSD %| 19           | 15            | 31           | 36            | 17           | 60            | 13           | 22            |
|      | p < 0.05     |               |              |               |              |               |              |               |
| Gly  | 97           | 134           | 142          | 120           | 229          | 283           | 217          | 283           |
| RSD %| 29           | 57            | 22           | 46            | 18           | 74            | 24           | 68            |
|      | p < 0.05     |               |              |               |              |               |              |               |
| Ala  | 117          | 160           | 176          | 157           | 221          | 194           | 201          | 181           |
| RSD %| 29           | 65            | 22           | 39            | 19           | 45            | 22           | 42            |
|      | p < 0.05     |               |              |               |              |               |              |               |
| Val  | 281          | 338           | 440          | 386           | 565          | 652           | 533          | 686           |
| RSD %| 19           | 50            | 17           | 34            | 17           | 52            | 19           | 50            |
|      | p < 0.05     |               |              |               |              |               |              |               |
| Met  | 130          | 110           | 160          | 133           | 204          | 231           | 195          | 218           |
| RSD %| 64           | 44            | 38           | 34            | 32           | 36            | 33           | 31            |
|      | p < 0.05     |               |              |               |              |               |              |               |
| Ile  | 242          | 293           | 387          | 355           | 495          | 626           | 467          | 596           |
| RSD %| 25           | 52            | 17           | 31            | 17           | 49            | 18           | 46            |
|      | p < 0.05     |               |              |               |              |               |              |               |
| Leu  | 398          | 440           | 558          | 496           | 651          | 757           | 593          | 697           |
| RSD %| 16           | 28            | 16           | 35            | 19           | 34            | 18           | 30            |
|      | p < 0.05     |               |              |               |              |               |              |               |
| Tyr  | 130          | 137           | 148          | 114           | 148          | 154           | 156          | 139           |
| RSD %| 45           | 75            | 35           | 41            | 34           | 57            | 36           | 67            |
|      | p < 0.05     |               |              |               |              |               |              |               |
| Phe  | 215          | 227           | 317          | 266           | 403          | 441           | 371          | 411           |
| RSD %| 22           | 26            | 17           | 34            | 14           | 26            | 16           | 23            |
|      | p < 0.05     |               |              |               |              |               |              |               |
| Lys  | 461          | 622           | 684          | 686           | 885          | 1257          | 845          | 1247          |
| RSD %| 19           | 48            | 19           | 37            | 21           | 50            | 22           | 50            |
|      | p < 0.05     |               |              |               |              |               |              |               |
| His  | 173          | 178           | 183          | 147           | 237          | 301           | 209          | 204           |
| RSD %| 31           | 39            | 29           | 36            | 22           | 47            | 58           | 41            |
arginine in sample C) to 1500 mg/100 g cheese (glutamic acid in samples B and D), demonstrating the presence of FAAs over a very wide range of concentrations (Table 3). On average, data obtained by IEC were about 7% lower than those obtained by HPLC, but were significantly less variable. For many individual FAAs, RSD values for HPLC data were almost twice as high as those for IEC. The higher variability of HPLC data is probably due to the instability of some OPA-amino acid derivatives (Heems et al. 1998). As an additional drawback, OPA reacts only with primary amines and hence does not allow detection of proline, which represents 8–10% of FAA in cheese. Due to these disadvantages and considering that concentrations of several FAAs were significantly different (p < 0.05) between the two techniques (Table 3), it was decided to perform the validation study only for the IEC method. A thorough investigation of the operating conditions of the seven laboratories using the IEC method and involved in the pilot test evidenced some relevant discrepancies in their calibration lines. As an example, calibration lines obtained for glutamine are shown in Fig. 2. The slope of calibration lines obtained by labs 2, 4, and 5 were very similar, and steeper than those of labs 3, 7, and 8. As a matter of fact, labs 3 and 8 were using instruments with poorly performing detectors and were asked to improve this aspect. Unexpectedly, lab 7 used an HPLC-IEC hybrid equipment, in-house modified for post-column derivatization with ninhydrin and, due to low sensitivity of the apparatus, doubled the concentration of standard solutions. This lab was excluded from participating to the validation study and replaced by another one. Furthermore, lab 6 used an injection volume of 20 μL (instead of 100 μL used at the other labs), resulting in very small, difficult to integrate peaks, and systematically produced the lowest data. This lab was invited to follow the provided protocol.

**Laboratory Training**

Prior to the collaborative study, a training day was organized for all participants, where the analytical procedure was shown and main critical steps were discussed. The major critical point was poor separation of partly overlapping peaks of asparagine, glutamic acid, and glutamine that could make the integration troublesome. Glutamic acid is more sensitive than asparagine and glutamine to changes in pH and elutes earlier when pH slightly increases. Optimum resolution is most easily obtained by adjusting the pH of eluting buffer 1 by 0.01–0.02 units. A typical IEC chromatogram of an amino acid standard is shown.

![Fig. 2 Calibration lines of glutamine obtained by IEC at different laboratories](image)

| Table 3 (continued) | Sample A | Sample B | Sample C | Sample D |
|---------------------|----------|----------|----------|----------|
|                     | IEC      | HPLC     | IEC      | HPLC     | IEC      | HPLC     | IEC      | HPLC     |
| Arg Mean            | 249      | 229      | 221      | 159      | 51       | 186      | 134      | 250      |
| RSD %               | 30       | 20       | 34       | 29       | 109      | 160      | 56       | 129      |

*Raw FAA data are expressed as mg/100 g cheese.
in Fig. 3, which also highlights a situation of poor peak resolution for the three mentioned FAA. It was furthermore necessary to substitute the isoleucine in the standard solution with one from a different producer, as the original gave a double peak in the chromatogram (not shown). All laboratories were informed about these aspects and requested to adopt decisive measures.

**Homogeneity and Stability Tests**

All the test samples passed the homogeneity and stability tests, carried out according to ISO Standard 13528:2015 (ISO 2015). Threshold values exceeding 0.3 were observed for tyrosine in samples 3 and 4, where its concentration was highest, probably because of the low solubility of this AA, which tends to crystallize in ripened cheeses (Tansman et al. 2015; D’Incecco et al. 2016b).

**Inter-Laboratory Validation Study**

Participants were asked to perform 12 determinations (three blind replicate analyses of four different cheese samples), in the minor possible lapse of time, and to return, together with their data, all of the obtained chromatograms, in order to detect problems which possibly occurred in separation. All laboratories were able to achieve optimal peak resolution and obtained calibration lines having $R^2 > 0.997$ for every FAA.

Statistical evaluation of the data and calculation of precision figures were carried out according to the internationally accepted procedures (ISO 2004) and are reported in Table 4. Considering the small number of participating laboratories, a 0.01 confidence level was adopted. In no case more than one laboratory was eliminated from the evaluation for the same FAA in the same sample; therefore, data from at least six laboratories were evaluated for every single amino acid in every single sample. Only 2% of the data were outliers and thus excluded from statistical evaluation. On the whole, these figures revealed a significant improvement if compared with those obtained in the pilot test (Table 3). This was the result of (1) availability of correctly performing equipment at all participating laboratories, (2) practical training, highlighting the critical points of the procedure, (3) strict application of the protocol, and (4) adoption of an internal standard.

The total amount of FAA determined in the four samples ranged between approx. 5500 and 8000 mg/100 g cheese, with a maximum relative standard deviation of repeatability ($RSD_r$) value of 2.7 and a maximum relative standard deviation of reproducibility ($RSD_R$) value of 5.6.

The mean content of single FAAs ranged from 8 mg/100 g (ornithine in sample 1) to 1380 mg/100 g (glutamic acid in sample 3), with a ratio which approximates 1:200. In about 75% of the cases, the average content of single FAAs fell in the range from 100 to 700 mg/100 g. The $RSD_r$ values were lower than 2.0 for 49 out of the 84 determined single FAA contents (58%). $RSD_r$ values exceeding 5.0 were observed only for FAAs present in the lowest amounts, i.e., glutamine, γ-aminobutyric acid, ornithine, or arginine. Indeed, these FAAs represent reagents or products of specific metabolic pathways of some LAB species, and their content gives interesting information (D’Incecco et al. 2016a; Brasca et al. 2016). $RSD_r$ values exceeding 5.0 were also observed for tyrosine, whose high $RSD_r$ values (sample 2 and sample 3) (already observed during the homogeneity test) are most likely due to low buffer pH.
### Table 4: Precision figures of individual FAA of the analyzed cheese samples determined by IEC within the validation study

| Amino acid | Sample 1 | | | Sample 2 | | | Sample 3 | | | Sample 4 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No. Labs | Mean | s | R | RSDR | No. Labs | Mean | s | R | RSDR | No. Labs | Mean | s | R | RSDR | No. Labs | Mean | s | R | RSDR | R |
| Asp | 7 | 139 | 0.8 | 0.61 | 2 | 13.4 | 9.66 | 38 | 7 | 208 | 2.5 | 1.19 | 7 | 13.9 | 6.69 | 39 | 7 | 261 | 6.7 | 2.55 | 19 | 23.3 | 8.93 | 49 |
| Thr | 6 | 209 | 1.8 | 0.88 | 5 | 12.0 | 5.75 | 34 | 6 | 245 | 3.6 | 1.49 | 10 | 4.7 | 1.92 | 13 | 7 | 183 | 4.3 | 2.34 | 12 | 11.0 | 5.99 | 31 |
| Ser | 7 | 272 | 3.1 | 1.14 | 9 | 18.9 | 6.94 | 53 | 7 | 371 | 6.3 | 1.70 | 18 | 21.2 | 5.71 | 60 | 7 | 450 | 10.7 | 2.37 | 30 | 30.2 | 6.71 | 85 |
| Asn | 6 | 275 | 4.7 | 1.71 | 13 | 7.4 | 2.70 | 21 | 6 | 321 | 3.6 | 1.11 | 10 | 5.1 | 1.59 | 14 | 7 | 336 | 10.1 | 3.00 | 28 | 24.0 | 7.14 | 68 |
| Glu | 7 | 892 | 9.4 | 1.05 | 26 | 32.1 | 3.59 | 90 | 7 | 1160 | 14.4 | 1.24 | 41 | 37.0 | 3.19 | 104 | 6 | 1382 | 20.1 | 1.46 | 57 | 46.8 | 3.39 | 132 |
| Gln | 7 | 135 | 6.2 | 4.59 | 17 | 13.2 | 9.77 | 37 | 7 | 52 | 3.8 | 7.26 | 11 | 7.6 | 14.64 | 22 | 7 | 61 | 5.3 | 8.75 | 15 | 9.6 | 15.90 | 27 |
| Gly | 7 | 115 | 1.5 | 1.33 | 4 | 8.7 | 7.51 | 24 | 7 | 155 | 1.8 | 1.14 | 5 | 9.9 | 6.40 | 28 | 7 | 237 | 5.6 | 2.38 | 16 | 14.4 | 6.08 | 41 |
| Ala | 7 | 142 | 1.6 | 1.16 | 5 | 12.4 | 8.75 | 35 | 7 | 178 | 1.6 | 0.89 | 4 | 10.5 | 5.92 | 30 | 7 | 200 | 5.3 | 2.65 | 15 | 14.3 | 7.15 | 40 |
| Cit | 7 | 58 | 1.5 | 2.59 | 4 | 5.4 | 9.43 | 15 | 6 | 287 | 3.0 | 1.04 | 8 | 9.9 | 3.46 | 28 | 7 | 299 | 9.1 | 3.04 | 26 | 20.3 | 6.79 | 57 |
| Val | 7 | 393 | 3.6 | 0.91 | 10 | 22.2 | 5.65 | 63 | 7 | 488 | 4.4 | 0.91 | 13 | 23.5 | 4.82 | 66 | 7 | 562 | 16.9 | 3.01 | 48 | 32.6 | 5.80 | 92 |
| Met | 7 | 123 | 1.6 | 1.35 | 5 | 7.6 | 6.24 | 22 | 7 | 156 | 1.8 | 1.18 | 5 | 12.1 | 7.72 | 34 | 7 | 193 | 8.3 | 4.31 | 23 | 15.7 | 8.14 | 44 |
| Ile | 7 | 349 | 3.9 | 1.12 | 11 | 17.1 | 4.90 | 48 | 6 | 424 | 4.9 | 1.17 | 14 | 5.4 | 1.28 | 15 | 7 | 496 | 18.0 | 3.63 | 51 | 30.4 | 6.13 | 86 |
| Leu | 7 | 565 | 2.9 | 0.52 | 8 | 38.8 | 6.87 | 109 | 7 | 662 | 6.4 | 0.97 | 18 | 24.0 | 3.62 | 68 | 7 | 669 | 27.3 | 4.08 | 77 | 50.4 | 7.53 | 142 |
| Tyr | 7 | 167 | 1.1 | 0.65 | 3 | 13.0 | 7.80 | 37 | 7 | 200 | 18.8 | 9.38 | 53 | 21.6 | 10.81 | 61 | 7 | 186 | 19.7 | 10.61 | 56 | 27.9 | 15.04 | 79 |
| Phe | 7 | 288 | 4.2 | 1.48 | 12 | 11.1 | 3.87 | 31 | 7 | 361 | 4.0 | 1.10 | 11 | 17.2 | 4.76 | 49 | 7 | 391 | 13.0 | 3.32 | 37 | 21.1 | 5.40 | 60 |
| Gaba | 7 | n.d. | – | – | – | – | – | – | 7 | 10 | 1.0 | 7.00 | 2 | 3 | 33.14 | 9 | 7 | 14 | 1.90 | 4 | 5 | 32.21 | 13 |
| Om | 7 | 8 | 0.2 | 3.10 | 1 | 2.0 | 25.56 | 6 | 7 | 25 | 0.5 | 1.89 | 1 | 3.1 | 12.22 | 9 | 7 | 38 | 1.0 | 2.69 | 3 | 3.6 | 9.44 | 10 |
| Lys | 6 | 629 | 6.3 | 1.00 | 18 | 45.2 | 7.19 | 127 | 8 | 700 | 7.7 | 0.98 | 22 | 30.8 | 3.95 | 87 | 7 | 928 | 23.4 | 2.53 | 66 | 46.3 | 4.99 | 131 |
| His | 7 | 204 | 2.3 | 1.13 | 6 | 12.9 | 6.32 | 36 | 7 | 232 | 3.1 | 1.33 | 9 | 16.6 | 7.18 | 47 | 7 | 218 | 7.3 | 3.33 | 21 | 15.9 | 7.26 | 45 |
| Arg | 6 | 246 | 2.6 | 1.05 | 7 | 4.8 | 1.97 | 14 | 7 | 33 | 1.5 | 4.73 | 4 | 6.0 | 18.15 | 17 | 7 | 24 | 1.8 | 7.49 | 5 | 4.7 | 19.53 | 13 |
| Pro | 7 | 538 | 9.4 | 1.75 | 27 | 45.7 | 8.50 | 129 | 7 | 663 | 10.2 | 1.54 | 29 | 30.6 | 4.62 | 86 | 6 | 707 | 22.9 | 3.24 | 65 | 28.1 | 3.98 | 79 |
| Total | 7 | 5760 | 40.8 | 0.71 | 115 | 296.8 | 5.15 | 837 | 6 | 6940 | 26.6 | 0.38 | 75 | 94.0 | 1.36 | 265 | 7 | 7860 | 208.6 | 2.65 | 588 | 440.0 | 5.60 | 1241 |

Raw FAA data are expressed as mg/100 g cheese

\* Below the quantification limit (0.1 mg/100 g cheese)
due to the low solubility of this AA, which is known to appear as white crystals in many types of ripened cheeses (Tansman et al. 2015; D’Incecco et al. 2016b).

For 71 out of the 84 determined single FAA contents (85%), the RSDR values were lower than 10.0, and values exceeding this level all referred to the same FAA with the lowest amounts above mentioned.

To further evaluate the results of the collaborative study, the obtained RSDR values were compared to those calculated by applying the Horwitz equation (Horwitz et al. 1980). For numerous analytes, a relationship exists between the measured mean concentration and its variability (RSDR), expressed by the equation:

\[
PRSDR = 2^{1 - 0.5 \log C}
\]  

Equivalent to

\[
PRSDR = 2^{-0.15 C}
\]

where \(C\) is the concentration of the analyte expressed as dimensionless mass fraction and PRSDR is the relative standard deviation under reproducibility conditions.

From this equation derives the Horwitz ratio (HorRat) (Horwitz and Albert 2006), which is the ratio of the RSDR calculated from the test data to the predicted RSDR (PRSDR) obtained by the Horwitz equation (2):

\[
\text{HorRat} = \frac{\text{RSDR}}{\text{PRSDR}}
\]

Under reproducibility conditions, HorRat values range between 0.5 and 2.0 (Horwitz and Albert 2006). Only in 12 out of 84 cases the HorRat values calculated for single FAAs in the four samples of this study (Table 5) exceeded the value of two, all referring to concentrations below 50 mg/100 g, and in 7 of these cases, HorRat did not reach the value of 3.0. The precision figures obtained in the present investigation are fully comparable to those reported in the AOAC Official Method 1994.12 (AOAC 1994) as well as to those indicated in Reg. (EC) No 152/2009 (European Commission 2009) for the determination of free lysine, methionine, and threonine in feeding stuffs.

As one of the aims of this study was to verify the possibility of applying the proposed IEC method to the quality control of different cheese types, the reliability of the proposed method was further checked by testing the capability to recognize authentic Grana Padano PDO cheese. The FAA data obtained for the test samples were evaluated according to a chemometric model we have recently developed for the characterization of Grana Padano PDO cheese. This model compares the relative content (i.e., expressed as percentage of total FAAs) of every single FAA of a cheese to the typical value, determined as the mean content in a set of 260 Grana Padano PDO samples of known age and origin. The differences between actual and expected values are expressed as Z-scores (number of standard deviations). In genuine Grana Padano PDO cheese, Z-score may exceed the value of 2.0 for a maximum of four single FAA, whereas only for one of these Z-score may exceed 3.0.

Figure 4 shows the evaluation of the data obtained at the participating laboratories for samples 1 and 3 according to the previously described model. The central solid line (Z-score = 0) indicates the typical mean value for each FAA, circles represent the average Z-score observed at the seven labs, and whiskers the range of variability. Sample 1, although produced adopting the traditional manufacturing process, was correctly recognized as a not authentic cheese, since it had not reached the minimum ripening period of 9 months. In fact, all laboratories certified Z-score over 2.0 for at least five different amino acids, all labs finding high contents for glutamine; asparagine and arginine, typical of young Grana Padano cheeses; and low values for glutamic and aspartic acid. On the contrary, sample 3 was recognized as a genuine Grana Padano PDO by all participating laboratories, since only for

### Table 5 HorRat values for individual FAA determined by IEC within the validation study

| Amino acid | Sample 1 | Sample 2 | Sample 3 | Sample 4 |
|------------|----------|----------|----------|----------|
| Asp        | 1.795    | 1.321    | 1.656    | 1.396    |
| Thr        | 1.136    | 0.389    | 1.128    | 1.157    |
| Ser        | 1.426    | 1.230    | 1.404    | 1.199    |
| Asn        | 0.555    | 0.334    | 1.483    | 1.307    |
| Gln        | 0.883    | 0.815    | 0.842    | 0.890    |
| Gly        | 1.807    | 2.307    | 2.572    | 2.462    |
| Ala        | 1.357    | 1.208    | 1.163    | 1.324    |
| Cit        | 1.630    | 1.142    | 1.381    | 1.610    |
| Val        | 1.535    | 0.716    | 1.413    | 1.418    |
| Met        | 1.227    | 1.082    | 1.306    | 0.378    |
| Ile        | 1.138    | 0.556    | 1.537    | 1.863    |
| Leu        | 1.046    | 0.282    | 1.289    | 1.364    |
| Tyr        | 1.575    | 0.851    | 1.683    | 1.401    |
| Phe        | 1.747    | 2.121    | 2.783    | 1.604    |
| Gaba       | 0.802    | 1.022    | 1.120    | 1.120    |
| Orn        | 3.091    | 1.757    | 1.443    | 3.231    |
| Lys        | 1.609    | 0.951    | 1.114    | 1.014    |
| His        | 1.245    | 1.440    | 1.395    | 1.662    |
| Arg        | 0.398    | 2.713    | 2.755    | 1.961    |
| Pro        | 1.935    | 1.087    | 0.874    | 1.365    |
| Total      | 1.745    | 0.455    | 1.835    | 1.560    |

*Below the quantification limit (0.1 mg/100 g cheese)
two FAAs (threonine and glycine) values just beyond the 2.0 Z-score limit were observed in a few laboratories.

Conclusions

The information achieved by determining 22 variables in a single analysis makes the evaluation of FAAs in cheese a powerful tool for studying the ripening and fermentation mechanisms and may allow to verify the authenticity of some PDO cheeses. However, analytical methods proposed so far for FAA determination by IEC have been validated at intra-laboratory level only, usually by evaluating day-to-day repeatability, making it difficult or even impossible to compare data from different labs. We have optimized a method for the determination of relevant FAAs in cheese, and the inter-laboratory study carried out to validate this method has demonstrated its adequacy for the quality control of cheese. The influence of instrumentation performances has been highlighted as well as the need for strict application of the analysis protocol to obtain reliable data.

On the basis of the results of this investigation, the validated method is currently applied for the determination of FAA patterns in the control of Grana Padano PDO cheese identity. We have recently adopted the proposed method for the FAA determination in other dairy products, including milk, fermented milk, infant formulae, milk-based beverages, and whey cultures, and proved it to be free of interference and to give the same performances as for cheese.

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Compliance with Ethical Standards

Conflict of Interest Johannes A. Hogenboom declares that he has no conflict of interest. Paolo D’Incecco declares that he has no conflict of interest.
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