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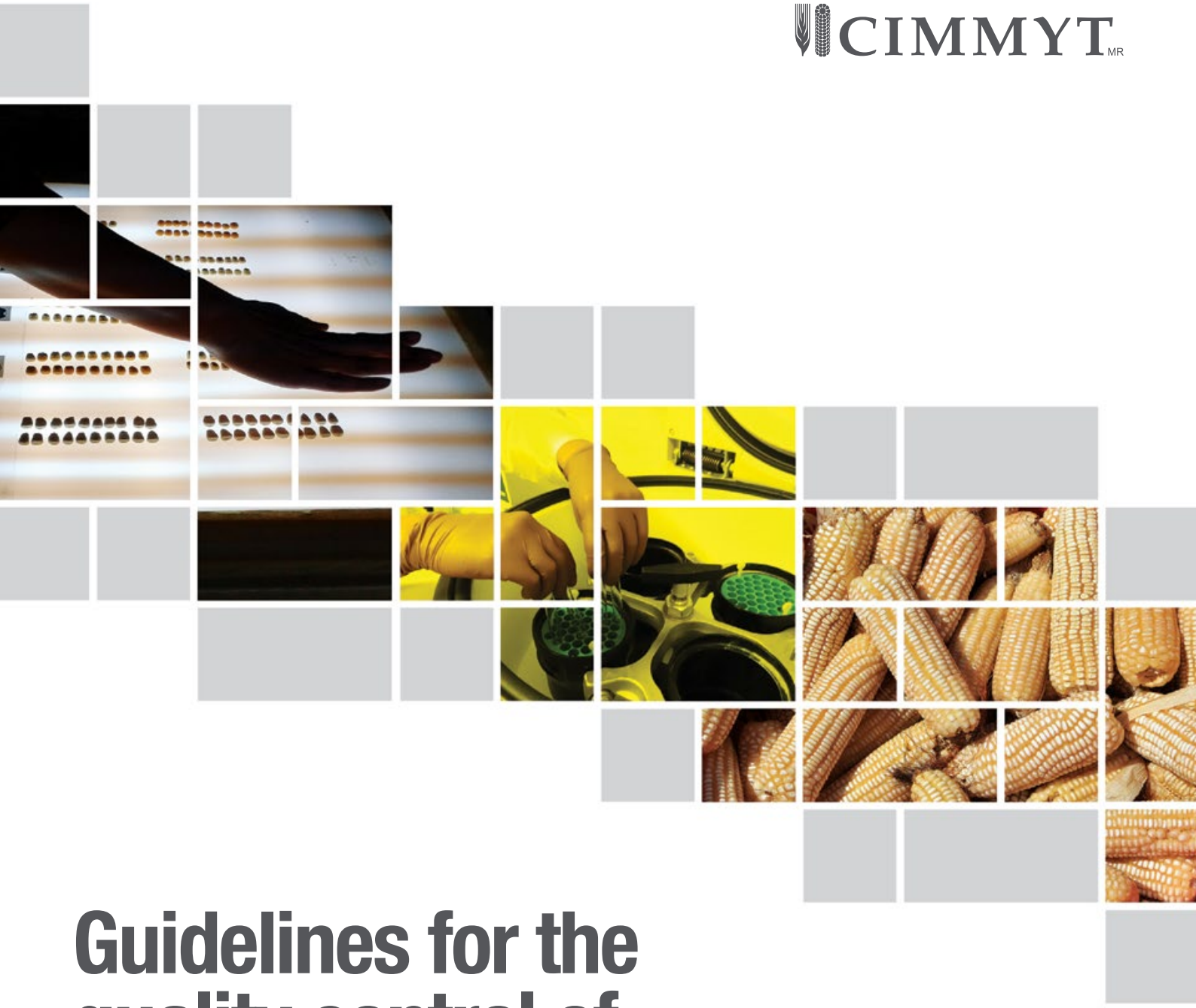

Guidelines for the quality control of Quality Protein Maize (QPM) seed and grain

Technical Bulletin

Strafford Twumasi-Afriyie, Natalia Palacios-Rojas, Dennis Friesen, Adefris Teklewold, Dagne Wegary, Hugo De Groote and B.M. Prasanna

CGIAR RESEARCH PROGRAM ON Maize

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CIMMYT MR
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CIMMYT - the International Maize and Wheat Improvement Center - is the global leader on publicly-funded maize and wheat research-for-development. Headquartered near Mexico City, CIMMYT works with hundreds of partners worldwide to sustainably increase the productivity of maize and wheat cropping systems, thus improving global food security and reducing poverty. CIMMYT is a member of the CGIAR Consortium and leads the CGIAR Research Programs on MAIZE and WHEAT. The Center receives support from national governments, foundations, development banks and other public and private agencies.

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Abstract: This guidebook is the first of its kind to provide recommendations and a framework for monitoring quality assurance of QPM. Guidelines are based on QPM genetics, breeding, characteristics, and nutritional benefits. It lists the QPM varieties based on CIMMYT germplasm that have been released worldwide. Furthermore, it elucidates the concepts of seed quality control, seed production and certification systems, definition of protein quality in QPM, methods for laboratory analysis of protein quality in QPM, sampling procedures for submitting samples to the lab, and sourcing a laboratory for analysis; it also gives recommendations for quality control in both QPM grain and recycled seed. This technical bulletin is intended to serve mainly as background information based on which to develop national QPM seed and grain quality control and assurance standards in countries where QPM is produced and marketed.

AAGROVOC Descriptors: Agricultural research; Agricultural engineering; Maize; Protein content; Quality controls; Seed production

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Acknowledgments

This technical bulletin on the quality control of quality protein maize (QPM) seed and grain was originally intended to serve as a background document for establishing QPM quality control standards in Ethiopia, as one of the deliverables under the Nutritious Maize for Ethiopia (NuME) project. Later it was decided to widen the scope of the bulletin, which now includes recommendations and a framework for quality assurance monitoring of QPM at the global level.

The work was initiated by the late Dr. Strafford Twumasi-Afriyie and was at an advanced draft stage when he passed away in 2013. We obviously want to credit him for his contributions and therefore have retained his name as first author of this publication. Twumasi devoted a significant part of his professional career to developing and deploying QPM in sub-Saharan Africa, with particular emphasis on Ghana and Ethiopia.

This technical bulletin on QPM was published with the support and contributions of several institutions and individuals. We sincerely acknowledge the funding support from Global Affairs Canada (GAC) for the NuME Project, and for making this publication possible. We also thank Drs. B.S. Vivek, Kevin Pixley and Amsal Tarekegne for reviewing and enriching the recommended scheme for QPM quality assurance monitoring and the framework that forms the core of this publication. We are also grateful to CIMMYT and IITA (the International Institute of Tropical Agriculture) colleagues who helped to review and define the list of QPM varieties released in different regions/countries. Particular thanks to Drs. Mosisa Worku in eastern and central Africa, Amsal Tarekegne in southern Africa, Baffour Badu-Apraku in West Africa, Daniel Paul Jeffers in China, B.S. Vivek in India and other south Asia countries, and Félix San Vicente and Luis Narro in Latin America. We also wish to extend our thanks to Aldo Rosales and all the staff of CIMMYT's Maize Nutritional Quality Laboratory who have been part and parcel of the historical progress of QPM and have contributed to the detailed protocols included here. All the previous work and published literature on QPM that served as the base for this technical bulletin are gratefully acknowledged. We thank CIMMYT Corporate Communications staff for their kind support in editing and designing the bulletin.

1. Introduction

Quality protein maize (QPM) belongs to a class of crops whose nutritional value has been enhanced through conventional breeding, a process known as biofortification (Bouis et al., 2011). QPM is characterized by a higher content of two essential amino acids, lysine and tryptophan (which are limited in common maize), and a hard, vitreous endosperm (Prasanna et al., 2001; Atlin et al., 2010). QPM is beneficial to consumers, especially young children and lactating women whose staple diet is dominated by maize and who have limited access to other protein sources. The main genetic system in QPM is *opaque2* (*o2*) (Gibbon and Larkins, 2005). The trait is conferred by the recessive allele o2 in homozygous state (*o2o2*), and unless proper precautions are taken, QPM varieties may lose the enhanced protein quality trait due to genetic contamination that results when QPM maize outcrosses with normal maize, which possesses the dominant allele *Opaque2* (*O2*). A quality control system is therefore needed to guarantee that the genetic purity and nutritional advantage of QPM is preserved during commercial seed multiplication/production as well as in QPM grain destined for commercial use and human/animal consumption.

Quality control for lysine and tryptophan contents, which are physically invisible traits, cannot be performed just by looking at the plant or the seed. Therefore, laboratory techniques are required to ensure that the benefits of QPM will reach the consumers.

The purpose of this document is to provide coherent quality control guidelines and appropriate standards for developing, producing and deploying QPM. These recommendations will be useful for institutions in various countries worldwide that are developing and commercializing QPM varieties. This document includes: (1) protocols to monitor the protein quality of QPM at various stages of breeding; (2) recommendations for recovering QPM quality following varietal contamination; (3) QPM quality standards for varietal release and registration; (4) quality standards for QPM seed production and marketing; and (5) quality standards for processing QPM grain for food/feed and for making commercial products.

2. Background

2.1. Development and characteristics of QPM

Development of enhanced protein quality in maize began in 1963 with the discovery of the nutritional benefits of a mutant maize gene, *o2*, which alters the protein composition and increases the lysine and tryptophan content in maize endosperm compared to conventional maize varieties that have the dominant allele (*O2*) at the same gene (Mertz et al., 1964; Prasanna et al., 2001). The better quality of the endosperm protein conferred by *o2* was attributed to the increased concentrations of two essential amino acids, lysine and tryptophan, which are limiting in conventional maize (Bressani, 1991; National Research Council, 1988).

Visually, maize kernels carrying the ***o2*** allele in homozygous state (*o2o2*) can be distinguished from conventional maize kernels because *o2o2* results in an endosperm that is soft, chalky and opaque to light transmission in contrast to conventional maize kernels (*O2O2* or *O2o2*) which are usually hard, vitreous and translucent. When maize kernels are spread on a light table,[1] opaque kernels can easily be separated from non-opaque ones (Vivek et al., 2008).

Although nutritionally superior, the soft, chalky consistency of *o2* maize kernels renders them susceptible to post-harvest insect pests and pathogens. To overcome these undesirable traits, maize breeders have exploited genetic variability for kernel hardness and chalkiness among *o2*-maize genotypes to select for *o2*-maize that is indistinguishable visually from conventional maize (Prasanna et al., 2001). Breeding for QPM involves manipulating three genetic systems: first is the ***o2*** gene which in its homozygous recessive state reduces the production of certain zein protein fractions that contain very low quantities of lysine and tryptophan, and increases the relative proportion of lysine- and tryptophan-rich non-zein fractions. Reduced zein protein content results in the undesirable soft and chalky (opaque) kernel features that are typical of *o2* maize. Second, endosperm modifier genes for the *o2* gene are visually selected to restore the hard, vitreous kernel features while maintaining high lysine and tryptophan concentration. The third system is the (non-*o2*) genetic system of amino acid modifiers affecting lysine and tryptophan

[1] A table with a translucent glass or plastic surface beneath which are fixed strong uniform lamps to give an illuminated surface.

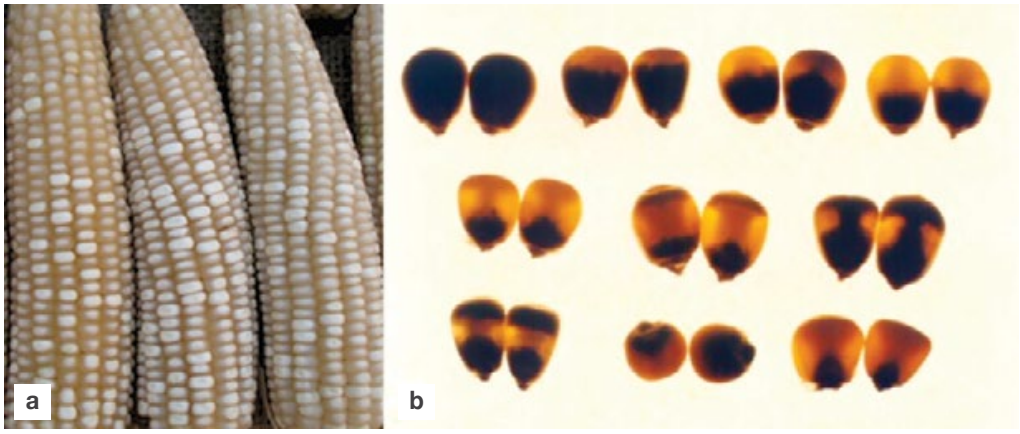

Figure 1. Maize ears and kernels carrying the *opaque 2 (o2)* allele: (a) ears segregating for chalky and normal kernels; and (b) various levels of kernel opaqueness in QPM kernels, as observed on a light table.

concentration in the endosperm that contributes to enhanced concentrations of these amino acids (Gibbon and Larkins, 2005; Krivanek et al., 2007).

Molecular marker-assisted selection is presently only possible for the first of the three genetic systems (Babu et al., 2005). Visual phenotypic selection using the light table helps to separate genotypes with modified endosperm (presence of the *o2* gene) and laboratory analyses are used to quantify and select for acceptable tryptophan and lysine concentrations (Vivek et al., 2008). The recessive nature of the *o2* coupled with its invisibility to the naked eye and the complexity of the endosperm modifier effects demand that care be taken to avoid contamination both in the production field and during post-harvest processing.

Currently there is no published literature on accepted QPM quality standards that would enable regulatory agencies, the seed industry, grain users (in particular, the food industry), the grain trade and other QPM users to properly judge the nutritional status of QPM varieties and monitor their protein quality.

2.2. Nutritional benefits of QPM

The proportionately higher kernel lysine and tryptophan contents of QPM varieties as compared to conventional maize provide a better balanced protein for humans and other monogastric animals such as pigs and poultry (Atlin et al., 2010; Bressani, 1991; National Research Council, 1988). Whereas the nutritional value of protein in conventional maize is approximately 40% that of milk, protein in QPM approaches 90%. Several nutritional studies with children and adults demonstrated the nutritional benefits of consuming QPM over conventional maize (Akuamoa-Boateng, 2002; Bressani, 1991; Brewster et al., 2005; Ortega Alemán et al., 2008; Rahmanifar and Hamaker, 1999). A recent meta-analysis of nine community-based studies (Gunaratna et al., 2010) indicated that consumption of QPM instead of non-QPM led to a 9% increase in the growth rate of young children and a 12% increase in the rate of weight gain in young children with mild to moderate under-nutrition in populations where maize is a significant part of the diet. In Ethiopia, a community-based study comparing the growth rate and weight gain of children under 5 years of age in households producing and consuming a QPM variety with households producing and consuming conventional maize found that the growth rate was 21% greater and weight gain was 26% greater in the QPM households (Akalu et al., 2010). Nuss and Tanumihadjo (2010) estimated that the target levels of QPM intake needed to meet lysine requirements are about 500 g for a 70-kg adult and about 100 g for a 12-kg child. This represents 40% reduction in maize intake relative to common maize to meet protein requirements.

Studies in Latin America and Africa (Ahenkora et al., 1999; De Groote et al., 2014; Pardo et al., 1972; Reyes-Moreno et al., 2003; Serna-Saldivar et al., 2008) indicated a preference for popular local food preparations using QPM over conventional maize. Double-blind consumer acceptance tests in East Africa found that consumers could identify and preferred traditional maize dishes prepared with QPM flour compared to the same dishes prepared with conventional maize (De Groote et al., 2014).

2.3. QPM varietal releases and adoption

Based on information accessed from different sources, more than 167 QPM varieties have so far been released worldwide (see Table 1). Regionally, more than half (53%) of the total QPM varietal releases happened in Africa, followed by Latin America (25%) and Asia (22%). Regarding the number of global QPM releases, South Africa leads with 26 varieties released (21 hybrids and 5 open-pollinated varieties) (Table 1). Varieties of the same pedigree are released in different countries with the same or different names. According to Krivanek et al. (2007), the QPM OPV (open-pollinated variety) Obatanpa has been released in as many as 15 sub-Saharan countries, and with the same name in 10 of the countries. About 58% of the QPM varieties released are hybrids.

Table 1. List of QPM varieties released till 2015 in Africa, Asia and Latin America.

Region	Country	Variety designation[1]	Variety type	Area of adaptation	Year of release
Africa	Ethiopia	BHQP542	Hybrid	Moist mid-altitude	2001
Africa	Ethiopia	Melkasa-6Q	OPV	Lo w moisture stress	2008
Africa	Ethiopia	BHQPY545	Hybrid	Moist mid-altitude	2008
Africa	Ethiopia	AMH760Q	Hybrid	Highland	2011
Africa	Ethiopia	MHQ138	Hybrid	Low moisture stress and moist mid-altitude	2012
Africa	Ethiopia	Melkasa-1Q	OPV	Low moisture stress	2013
Africa	Ethiopia	BHQPY548	Hybrid	Moist mid-altitude	2015
Africa	Kenya	WSQ104	OPV	Mid altitude	2000
Africa	Kenya	KH500Q	hybrid	Mid altitude	2003
Africa	Kenya	KH631Q	hybrid	Mid altitude	2003
Africa	Kenya	Kh531Q	hybrid	Transition highland	2004
Africa	Tanzania	LISHE H1	hybrid	Mid altitude	2003
Africa	Tanzania	LISHE H2	hybrid	Mid altitude	2003
Africa	Tanzania	TANH611	hybrid	Mid altitude	2006
Africa	Tanzania	NATAH6Q	OPV	Mid altitude	2013
Africa	Tanzania	MAMS H0913	hybrid	Mid altitude	2014
Africa	Uganda	Longe-5	OPV	Mid altitude	2000
Africa	Uganda	NALONGO	OPV	Mid altitude	2002
Africa	Uganda	SALONGO	Hybrid	Mid altitude	2008
Africa	Uganda	VP Max	OPV	Mid altitude	2012
Africa	South Sudan	Longe-5	OPV	Mid altitude	Informal
Africa	Mozambique	Sussuma	OPV	Medium-to-high altitude	2003
Africa	Mozambique	OLIPA	Hybrid	Medium-to-high altitude	2008
Africa	Malawi	Chitedze 2	OPV	Mid altitude	2008
Africa	Malawi	MH29	hybrid	Mid altitude	2009
Africa	South Africa	QS7608	Hybrid	Wet & dry mid-altitude	1996
Africa	South Africa	QS7701	Hybrid	Wet & dry mid-altitude	1996
Africa	South Africa	QS-7705	Hybrid	Wet & dry mid-altitude	1997
Africa	South Africa	QS 7606	Hybrid	Wet & dry mid-altitude	1997
Africa	South Africa	QS7705	Hybrid	Wet & dry mid-altitude	1997
Africa	South Africa	QS7703	Hybrid	Wet & dry mid-altitude	2001
Africa	South Africa	QS7707	hybrid	Wet & dry mid-altitude	2002
Africa	South Africa	QS7709	Hybrid	Wet & dry mid-altitude	2002
Africa	South Africa	QS7614	Hybrid	Wet & dry mid-altitude	2003
Africa	South Africa	QS7616	Hybrid	Wet & dry mid-altitude	2003
Africa	South Africa	QS7646	hybrid	Wet & dry mid-altitude	NA[2]
Africa	South Africa	QS7715	hybrid	Wet & dry mid-altitude	2004
Africa	South Africa	QS7751	hybrid	Wet & dry mid-altitude	2005
Africa	South Africa	QS7761	hybrid	Wet & dry mid-altitude	2005
Africa	South Africa	QS7717	hybrid	Wet & dry mid-altitude	2006
Africa	South Africa	QSOba	OPV	Mid altitude	2006
Africa	South Africa	Obatanpa SR	OPV	Mid altitude	2008
Africa	South Africa	QS-king	OPV	Mid altitude	2009
Africa	South Africa	QS-Mini	OPV	Mid altitude	2010
Africa	South Africa	Nelson's choice QPM	OPV	Mid altitude	2012
Africa	South Africa	CAP9006QS	hybrid	Wet & dry mid-altitude	2012
Africa	South Africa	CAP9444NG	hybrid	Wet & dry mid-altitude	2013
Africa	South Africa	CAP9015	hybrid	Wet & dry mid-altitude	2014
Africa	South Africa	Q623	Hybrid	Wet & dry mid-altitude	2014
Africa	South Africa	SA4115Q	hybrid	Wet & dry mid altitude	2015
Africa	South Africa	JEMAT601Q	hybrid	Wet & dry mid altitude	2015
Africa	Zambia	Obatanpa	OPV	Mid altitude	2004
Africa	Zambia	GV687P	hybrid	Mid altitude	2015
Africa	Zambia	GV682P	Hybrid	Mid altitude	2015
Africa	Zimbabwe	Obatanpa	OPV	Mid altitude	2003
Africa	Zimbabwe	ZS261Q	Hybrid	Mid Altitude	2006
Africa	Zimbabwe	MQ623	Hybrid	Wet & dry mid-altitude	2014
Africa	Lesotho	VP05120	OPV	Wet & dry mid-altitude	2011
Africa	Burkina Faso	Espoir	OPV	Guinea Savanna, Sudan savanna	2004
Africa	Ghana	Obatanpa	OPV	Forest, Forest-savanna transition, Guinea savanna, Derived savanna zones	1992
Africa	Ghana	Daba-ba	Hybrid	Forest, Forest-savanna transition, Guinea savanna, Derived savanna zones	1997
Africa	Ghana	Cida-ba	Hybrid	Forest, Forest-savanna transition, Guinea savanna, Derived savanna zones	1997

Table 1. List of QPM varieties cont'd.

Region	Country	Variety designation[1]	Variety type	Area of adaptation	Year of release
Africa	Ghana	Mamaba	Hybrid	Forest, Forest-savanna transition, Guinea savanna, Derived savanna zones	1997
Africa	Ghana	Golden Jubilee	OPV	Forest, Forest-savanna transition, Guinea savanna, Derived savanna zones	2007
Africa	Ghana	Etubi	Hybrid	Forest, Forest-savanna transition, Guinea savanna, Derived savanna zones	2007
Africa	Ghana	Akposoe	OPV	Forest, Forest-savanna transition, Guinea savanna, Derived savanna zones, Sudan savanna	2007
Africa	Ghana	Aziga	OPV	Forest, Forest-savanna transition, Guinea savanna, Derived savanna zones	2007
Africa	Ghana	Omankwa	OPV	Forest, Forest-savanna transition, Guinea savanna, Derived savanna zones, Sudan savanna	2010
Africa	Ghana	Abontem	OPV	Forest, Forest-savanna transition, Guinea savanna, Sudan savanna, Derived savanna zones	2010
Africa	Ghana	Enibi	Hybrid	Forest, Forest-savanna transition, Guinea savanna, Derived savanna zones	2010
Africa	Ghana	Aburohemaa	OPV	Forest, Forest-savanna transition, Guinea savanna, Derived savanna zones, Sudan savanna	2010
Africa	Nigeria	Obatanpa (Sammaz 14)	OPV	Forest, Forest-savanna transition, Guinea savanna, Sudan savanna	2005
Africa	Nigeria	EV 99 QPM	OPV	Forest, Forest-savanna transition, Guinea savanna, Sudan savanna	2010
Africa	Mali	Obatanpa	OPV	Guinea savanna, Sudan savanna	1998
Africa	Senegal	EV 99 QPM	OPV	Guinea savanna, Sudan savanna	1998
Africa	Senegal	DMR ESR W QPM	OPV	Guinea savanna, Sudan savanna	1998
Africa	Senegal	Susuma (Obatanpa)	OPV	Guinea savanna, Sudan savanna	2000
Africa	Togo	Obatanpa	OPV	Forest, Forest-savanna transition, Guinea savanna, Sudan savanna	2003
Africa	Togo	EV 99 QPM	OPV	Forest, Forest-savanna transition, Guinea savanna, Sudan savanna	2003
Africa	Benin	Houlin-mi or Faaba (Obatanpa)	OPV	Forest, Forest-savanna transition, Guinea savanna,	1994
Africa	Cameroon	Obatanpa	OPV	Forest, Guinea savanna,	2003
Africa	Cote d'Ivoire	Obatanpa	OPV	Forest, Guinea savanna,	2002
Africa	Cote d'Ivoire	EV 99 QPM	OPV	Forest, Forest-savanna transition, Guinea savanna,	Informal
Africa	Cote d'Ivoire	DMR ESR W QPM	OPV	Forest, Forest-savanna transition, Guinea savanna,	Informal
Africa	Guinea	K9101	OPV	Forest, Guinea savanna	1992
Africa	Guinea	Obatanpa	OPV	Forest, Guinea savanna	1997
Africa	Guinea	CMS 475	OPV	Forest, Guinea savanna	1999
Africa	Guinea	CMS 473	OPV	Forest, Guinea savanna	NA
Africa	Guinea	BR473	OPV	Forest, Guinea savanna	NA
LA[3]	Bolivia	Aychasara 2	OPV	Lowland tropics	2009
LA	Bolivia	INIAF-HQ2	Hybrid	Lowland tropics	2013
LA	Colombia	SV7048	Hybrid	Lowland tropics	2009
LA	Colombia	FNC31AC	OPV	Mid altitude	2010
LA	Colombia	FNC32AC	OPV	Mid altitude	2010
LA	Colombia	ICA V159	OPV	Lowland tropics	2013
LA	Colombia	CORPOICA H112	Hybrid	Lowland tropics	2002
LA	Costa Rica	UPIAV-G6	OPV	Lowland tropics	2006
LA	Costa Rica	PROTEINTA	OPV	Lowland tropics	2009
LA	Costa Rica	NUTRIGRANO	OPV	Lowland tropics	2011
LA	El Salvador	Oro Blanco	Hybrid	Lowland tropics	2008
LA	El Salvador	Platino	Hybrid	Lowland tropics	2009
LA	El Salvador	PROTEMAS	OPV	Lowland tropics	2009
LA	El Salvador	Dorado	OPV	Lowland tropics	2013
LA	Peru	Inia611 NutriPeru	Hybrid	Lowland tropics	2010
LA	Guatemala	HRQ-2988	Hybrid	Lowland tropics	2010
LA	Guatemala	HRQ-596	Hybrid	Lowland tropics	2011

Table 1. List of QPM varieties cont'd.

Region	Country	Variety designation[1]	Variety type	Area of adaptation	Year of release
LA	Guatemala	ICTA MAYA	Hybrid	Lowland tropics	2012
LA	Haiti	HUGO	OPV	Lowland tropics	2008
LA	Honduras	DICTAHQ10	Hybrid	Lowland tropics	2009
LA	Honduras	Sintetico 03	OPV	Lowland tropics	2010
LA	Honduras	Lempira QPM	OPV	Lowland tropics	2013
LA	Mexico	V556AC	OPV	Lowland tropics	2008
LA	Mexico	H519C	Hybrid	Lowland tropics	2008
LA	Mexico	H564C	Hybrid	Lowland tropics	2010
LA	Mexico	ZAPATA 3	Hybrid	Subtropical	2010
LA	Mexico	ZAPATA 9	Hybrid	Subtropical	2010
LA	Mexico	QPM H-373 C	Hybrid	Subtropical	2011
LA	Mexico	RY800	Hybrid	Lowland tropics	2011
LA	Nicaragua	NUTRINTA Amarillo	OPV	Lowland tropics	2005
LA	Nicaragua	NUTRADER	OPV	Lowland tropics	2006
LA	Nicaragua	INTA SEQUIA QPM	OPV	Lowland tropics	2014
LA	Nicaragua	Mazorca de oro doble	Hybrid	Lowland tropics	2015
LA	Panama	IDIAPMQ-02	OPV	Lowland tropics	2008
LA	Panama	IDIAPMQ-07	OPV	Lowland tropics	2008
LA	Panama	IDIAPMQ-12	OPV	Lowland tropics	2009
LA	Panama	IDIAPMQ-14	OPV	Lowland tropics	2009
LA	Panama	IDIAPMQ-09	OPV	Lowland tropics	2013
LA	Venezuela	INIAQPM2	Hybrid	Lowland tropics	2007
LA	Venezuela	INIAQPM28	Hybrid	Lowland tropics	2008
LA	Venezuela	INIASQ1	OPV	Lowland tropics	2009
LA	Venezuela	INIASQ2	OPV	Lowland tropics	2009
Asia	Bhutan	Chaskarpa	OPV	Mid-hills	2012
Asia	Bhutan	Shafangma Ashom	OPV	Mid-hills	NA
Asia	China	Yunrui-1	Hybrid	Subtropical	2010
Asia	China	Yunrui-8	Hybrid	Subtropical	2010
Asia	China	Zhongdan 9409	Hybrid	Temperate	2002
Asia	China	Zhongdan 3850	Hybrid	Temperate	2000
Asia	China	Yun Yao 19	Hybrid	Subtropical	2002
Asia	China	Yun You 167	Hybrid	Subtropical	2002
Asia	China	Lu Dan 206	Hybrid	Temperate	2001
Asia	China	Lu Dan 207	Hybrid	Temperate	2001
Asia	China	Lu Dan 203	Hybrid	Temperate	1999
Asia	China	Lan Dan807	Hybrid	NA	NA
Asia	China	Hybrid 2075	Hybrid	NA	NA
Asia	China	Yumietou 102	Hybrid	Tropical	2006
Asia	India	Shakti	OPV	Tropical rainfed	1971
Asia	India	Rattan	OPV	Tropical rainfed	1971
Asia	India	Shakti 1	OPV	Tropical rainfed	1997
Asia	India	Protina	OPV	Tropical rainfed	1997
Asia	India	Shaktiman 1	Hybrid	Tropical rainfed	2001
Asia	India	Shaktiman 2	Hybrid	Tropical rainfed	2004
Asia	India	HQPM 1	Hybrid	Tropical rainfed	2006
Asia	India	Shaktiman 3	Hybrid	Tropical rainfed	2006
Asia	India	Shaktiman 4	Hybrid	Tropical rainfed	2006
Asia	India	HQPM 5	Hybrid	Tropical rainfed	2007
Asia	India	HM 8	Hybrid	Tropical rainfed	2007
Asia	India	HQPM 7	Hybrid	Tropical rainfed	2008
Asia	India	Vivek QPM 9	Hybrid	Mid-Altitude/Highland, rainfed	2008
Asia	India	HM 10	Hybrid	Tropical rainfed	2008
Asia	India	HM 11	Hybrid	Tropical rainfed	2009
Asia	Indonesia	Srikandi Putih-1	OPV	Lowland tropics	2004
Asia	Indonesia	Srikandi Kuning-1	OPV	Lowland tropics	2004
Asia	Indonesia	BIMA 12Q	HYbrid	Lowland tropics	2011
Asia	Indonesia	BIMA 12Q	HYbrid	Lowland tropics	2011
Asia	Nepal	Poshilo Makai-1	OPV	Mid-hills	2008
Asia	Vietnam	HQ 2000	Hybrid	Lowland tropics	NA
Asia	Vietnam	LCH9	Hybrid	Lowland tropics	NA

[1] The list of QPM varieties released in the three continents may not be exhaustive.
[2] NA = Not available. [3] LA = Latin America.

Adoption of QPM, however, has been considerably less widespread. It is estimated that by 2015, about 1 million ha of land were covered with QPM in Sub-Saharan Africa, with Ghana and Uganda together accounting for ~50% of this, and 18 other countries accounting for the rest. It is estimated that about 150,000 ha of land are covered by QPM in 12 Latin American countries, mostly in Venezuela, Guatemala, Mexico, Honduras, Bolivia and Colombia. Approximately 250,000 ha are sown to QPM in Asia, with 80-90% of this area in selected provinces of China and the rest in India, Nepal, Philippines and Indonesia.

Ghana and Uganda are among the countries where QPM has been widely adopted. This success can be largely attributed not only to the superior agronomic performance (grain yield, in particular) of QPM varieties and their associated nutritional benefits and consumer acceptance, but also to the involvement of governmental entities in promoting, popularizing and disseminating QPM.

Atlin et al. (2010) recognized two types of maize farmers: (1) farmers who produce for the commercial grain market, and (2) farmers who produce, often on a subsistence basis, maize grain for home consumption or the local trade. The authors suggested the following possible reasons for poor adoption of QPM despite the availability of numerous superior QPM varieties and the substantial efforts by the public sector to promote them. The challenges facing the first type of farmer include the invisibility of the QPM trait, which makes QPM grain visually indistinguishable from conventional maize grain. This renders QPM vulnerable to adulteration, particularly where it commands a premium price, and makes it necessary to market it through a dedicated supply chain.

Breeding QPM germplasm is expected to be more costly than breeding conventional maize for the following reasons:

i. The frequency of recovering genotypes with *opaque2* (o2o2) homozygous allele is only 25% in the F2 generation;

ii. The probability of obtaining genotypes with a hard and vitreous kernel phenotype within the *opaque2* segregating families is low;

iii. Accumulating amino acid modifier genes that ensure the desired levels of lysine and tryptophan in a particular genotype requires additional breeding efforts;

iv. Laboratory determination of kernel tryptophan and lysine contents in breeding materials needs to be done at least twice (the cost of determining the kernel protein, tryptophan and lysine contents in a sample is about US$ 18) along the breeding pipeline for variety registration/release.

Therefore, the added cost of breeding QPM varieties as compared to the conventional maize varieties implies that QPM grain must either command a premium price on the market to offset the cost of seed or, where that is not possible, the cost of QPM seed may need to be subsidized. The subsidy may also take the form of a government-supported QPM breeding program. This may be attractive to governments or agencies that wish to improve the protein nutrition of poor rural populations dependent on maize as their staple cereal. Therefore, QPM technology is primarily a stop-gap means of improving the nutritional status of subsistence rural populations until their economic status improves sufficiently for them to afford a higher quality diet.

3. Seed Quality Control

Seed quality control usually takes place at two levels: (1) internal quality control, performed at the seed company level, whether private or public, and (2) external quality control, carried out by a governmental or autonomous regulatory agency, usually referred to as a seed certification unit. External quality control is generally the prerogative of sovereign national bodies, some of which are affiliated to regional and international bodies such as the Organization for Economic Cooperation and Development (OECD), the Food and Agricultural Organization (FAO) of the United Nations or the Union of Plant Variety Protection (UPOV). Organizations such as the International Seed Testing Association (ISTA) (a collaboration of seed scientists and seed analysts from universities, research centers, and private and government seed testing laboratories around the world) develop, adopt and publish standard procedures for sampling and testing seeds, and promote uniform application of these procedures for evaluating seeds moving in international trade (ISTA, 2014).

Seed quality control is not just a laboratory exercise; as a living biological material, the viability and vigor of a seed is affected by the conditions from the time its parents are sown until it is itself sown by the farmer. Thus, seed quality control must be a part of each stage of the seed production process (MacRobert, 2009). Key aspects that must be considered include:

- Assurance that the source seed is pure and of the highest quality;
- Selection and management of seed production fields in which the seed crop is adequately watered and nourished to provide optimal growth conditions;
- Especially in hybrid seed production, careful planting of male and female parents, roguing of off-types, timely and complete tassel removal from female parents, and removal of male parents after pollination;
- Careful and timely harvesting of the seed at physiological maturity, and drying (when necessary) to 13% moisture content to minimize deterioration in storage;
- Processing to remove impurities and inferior seeds, and treatment to prevent fungal infection and insect attack;
- Storage at cool temperatures and low relative humidity to minimize deterioration;
- Careful control of inventory to ensure that stocks are rotated through the warehouse in a timely manner and are not allowed to deteriorate as a consequence of excessive time in storage;
- Testing to assess and/or confirm whether the seed meets standards of purity, germination percentage, cleanliness, absence of disease, and other desirable or undesirable traits.

3.1. Seed production and certification systems

The purpose of seed certification is to maintain and preserve genetic purity and identity of released varieties as described and confirmed by an official variety release agency. Rules and regulations governing seed certification are established by national regulatory bodies and usually conform to the international standards described, for example, by ISTA (ISTA, 2014). They define standards and specifications for both production and physical purity, including growing conditions, isolation, inspection, presence of weed seeds, defective seeds, germination percentage and moisture content, all determined by established and accepted methodologies including sampling methods (MacRobert, 2009).

The different classes of maize seed, their maintenance and production were described by MacRobert (2009). Maize seeds are maintained in three major classes before reaching the farmer, namely: breeder seed,

foundation (or pre-basic and basic) seed and certified seed (CIMMYT, 1984). Breeder seed is produced by the breeder of the cultivar from progenitors of distinct genetic stock. The breeder or organization holding breeders' rights over a released cultivar is responsible for maintaining the breeder seed and ensuring that its genetic purity is conserved. The first increase of breeder seed is usually referred to as foundation or basic seed. Usually agencies other than those holding the breeders' rights are responsible for maintaining and multiplying foundation seed. Certified seed is the last stage of seed multiplication before it reaches the farmer for planting and grain production. Certified seed is produced by the foundation seed agency or other third parties at the discretion of seed governing bodies. In an organized seed production program, certified seed should always be produced by a separate department or unit, depending on the type of organization – private or public – within the seed industry. At all levels of seed production, morphological traits of the plants and ears selected to form part of the seed lots should meet standards prescribed by seed laws and regulations based on a varietal description provided by the breeder/owner of the variety.

3.2. QPM Quality assurance

In addition to the quality assurance standards applied during conventional maize seed production, nutritionally enhanced food crops, such as QPM, require standards of nutritional quality based on acceptable prescribed levels of the relevant nutritional trait for seed production and certification (Basra, 1995; McDonald, 1998; Morris and Smale, 1997; Tripp, 2001; Tripp and Louwaars, 1997; Tripp et al., 2007; Tripp and Rohrbach, 2001).

First, it is important to ensure that the protein content of the sample is within the range of any maize (8 to 11%). Then, the levels of kernel tryptophan and lysine need to be monitored. Lysine levels in protein of conventional and QPM maize average 2.0% and 4.0%, respectively, but depending on the genetic background, they may range from 1.5-2.8% and 2.6-5.0%, respectively. Tryptophan levels in kernel protein of conventional and QPM maize usually average 0.5% and 0.8%, respectively (Moro et al., 1996; Vivek et al., 2008). The levels of tryptophan and lysine in maize are highly correlated (>0.9) and, since analysis for tryptophan is easier and less expensive, breeders typically use tryptophan levels as an indicator of the nutritive value of a QPM variety (Nurit et al., 2009; Villegas et al., 1992).

There are no absolute kernel lysine or tryptophan values that define QPM (Vivek et al., 2008); however, a scale (presented in Table 2) has been suggested for classifying QPM and non-QPM materials. The definition of QPM can also be expressed in terms of a quality index (QI), which is the tryptophan-to-protein ratio in the sample, and expressed as a percentage:

$$QI = 100 \times \frac{\text{percentage of tryptophan in the sample}}{\text{percentage of protein in the sample}}$$

It is commonly accepted that the QI must be higher than 0.8 to classify the grain as QPM.

3.2.1. Laboratory analysis of QPM protein quality

Various laboratory protocols are available for determining protein-bound lysine and tryptophan content in maize grain, including high performance liquid chromatography (HPLC) (Huang et al., 2006), bacterial assays (Scott et al., 2004) and colorimetric reactions (Nurit et al., 2009; Villegas et al., 1984; Vivek et al., 2008). More recently, near infrared reflectance spectroscopy (NIRS) has been applied to estimate both crude protein and lysine and tryptophan content in maize grain (Rosales et al., 2011). The choice of a laboratory method depends on the purpose of the analysis, the degree of accuracy required, and the cost. Lysine and/or tryptophan determination by HPLC-based

Table 2. Kernel lysine and tryptophan levels as percentages of total protein and sample in whole grain flour of normal and *o2o2* maize.

	Amino acid	QPM		Non-QPM
In protein	Lysine (%)	>4		<3.5
	Tryptophan (%)	>0.8		<0.50
In sample		*Whole grain*	*Endosperm*	*Whole grain*
	Lysine (%)	>0.35	>0.32	<0.32
	Tryptophan (%)	>0.075	>0.07	<0.65

Modified from Vivek et al., 2008.

amino acid analysis is the most costly (typically in excess of US$ 100 per sample) and can be time-consuming (at least two days per sample). It is, therefore, not suited for routine analysis and should be reserved only to confirm amino acid content when registering a new QPM variety, and performed by an accredited laboratory offering amino acid analysis, preferably on a commercial basis.[2]

A rapid, relatively inexpensive colorimetric method for tryptophan determination was developed by CIMMYT for use in its QPM breeding program in the early 1960s (Villegas et al., 1984). The method is based on the reaction of one molecule of glyoxylic acid with two molecules of tryptophan following defatting of the maize endosperm and enzymatic hydrolysis of the proteins with papain. In the original method, glyoxylic acid was delivered as an impurity in acetic acid and the efficacy of the method was thus dependent on the purity of the acetic acid batch used. Consequently, transfer of the method to collaborating laboratories that did not have access to the same reagent suppliers as CIMMYT resulted in inconsistent and often poor application of the method. To solve this problem, a modification of the "acetic acid method" was developed in which glyoxylic acid was explicitly introduced into the original methodology (Nurit et al., 2009). The modified "glyoxylic acid method" delivers more reproducible and consistent results. Total crude protein in the sample is quantified separately using standard micro-Kjeldahl digestion followed by colorimetric quantitation of total N in the digestor on an auto-analyzer (Galicia et al., 2008; Nkonya et al., 1997). Table 3 presents the current cost of the analysis at CIMMYT's Maize Quality Laboratory in Mexico.

The NIRS method offers many advantages over the wet chemistry colorimetric methods, which require considerable time-consuming sample preparation including grinding, defatting and enzymatic hydrolysis prior to tryptophan analysis. This method is about 65% cheaper and 99% faster than the wet chemistry analysis. Calibration curves of NIRS versus wet chemistry determinations for protein-bound tryptophan and lysine as well as crude protein in maize kernels have been developed at CIMMYT and are currently being used by CIMMYT and partners for analyzing the quality of QPM breeding materials in early generations (Rosales et al., 2011) The cost of a determination (in which lysine, tryptophan and crude protein are determined simultaneously from a single sample) is currently about US$ 2 per sample. See Table 4 for a comparison of the costs of colorimetric and NIRS-based methods.

[2] For example, University of Missouri Agriculture Experiment Station Chemical Laboratories, Columbia, MO 65211-7170, USA.

Table 3. Time required and cost of major activities for determination of protein, tryptophan and lysine contents by the wet chemistry method.

Sample preparation	Units to report	Brief description	Sample preparation cost (US$)
Grinding (Cyclonic mill)	N/A	General grinding (at least 30 seeds)	2.10
Defatting samples (required for several analyses)	N/A	Necessary to eliminate the fat, as it can interfere during chemical reactions	2.10
Determining nitrogen and % of protein (dry weight basis)	%	Analysis of nitrogen content expressed on a dry weight basis	5.70
Determining kernel tryptophan content	%	Colorimetric method; this analysis is performed in whole maize kernel or endosperm	3.60
Determining kernel lysine content	%	Colorimetric method; this analysis is performed in whole maize kernel or endosperm	4.60

Table 4. Description and cost of major activities for the determination of protein, tryptophan and lysine contents by NIRS.

Activities to be performed		Glyoxylic method	NIR	Cost (US$)
Sample preparation	Grinding	2 min	2 min	2
	Defatting	10 h	0 h	2
Protein hydrolysis		18 h	0 h	1
Sample analysis	Chemical reaction and calculations	1 h	0 h	2
	Sample scanning in NIRS	0 min	1 min	0.5

NIRS methodology is recommended to screen early generation breeding samples. The colorimetric method (glyoxylic acid method) must be used for advanced generation breeding materials and pre-commercial samples. The lab protocols for the colorimetric and NIRS methods are included in Annex 1 and 2, respectively.

3.2.2. Sampling procedure for submitting samples to the laboratory

Accurate analytical information on quality is vital for successfully selecting and commercializing varieties (Smith et al., 2012). The sampling methodology and experimental design used to phenotype many quality traits will depend on the purpose of the study, number of samples, cost of the analysis, etc. Quality traits can be measured through single-phase experiments where varieties are grown without field replicates and one sample is taken for laboratory analysis, or through multi-phase experiments in which varieties are first grown in a replicated field trial and then further processed in a laboratory. The common practice is to test a single field replicate for each variety or a single composite sample formed by combining grain samples before submitting them to the laboratory or in the laboratory (Smith et al., 2012). Such an approach may be applied for commercial varieties or advanced breeding materials, where the traits to be analyzed are fixed. For early-generation breeding samples, other approaches can be followed, such as partial replication, where a subset of the varieties is tested using multiple field replicate samples, and then a subset of the selected field plots is split to produce replicate samples for the laboratory analysis. A similar approach can be used in single-phase experiments, where some varieties are tested using individual replicate samples and others are tested using composite samples (Smith et al., 2012). It is therefore very important to discuss the purpose of the study with the laboratory analyst and come to an agreement on a suitable sampling process to reduce error and, possibly, the cost.

In addition to the above methodologies, here are some important general considerations when sending samples for protein quality analysis:

1. Self-pollinate the plants that you are planning to use for quality analysis.
2. If possible, only sample kernels from the central part of the ears.
3. For OPVs, send at least 500 g per sample to the lab.
4. For early-generation materials, send at least 100 kernels per sample.
5. For advanced lines and hybrids, send at least 50 kernels per sample.

However, when sampling certified QPM seed lots, you should follow standards and procedures established by the seed regulatory bodies in the respective countries, and/or ISTA (2014) guidelines.

3.2.3. Sourcing a laboratory for quality analysis

Some important points to consider when choosing the laboratory that will perform the quality analysis are:

1. Management of quality assurance in the lab: traceability of the sample throughout the process, traceability of the results (certification of the lab, use of standard reference materials, participation in inter-laboratory assessments).
2. Generation of accurate results from your samples: to assess the quality of laboratory outputs, you can mix samples thoroughly and send subsamples to a laboratory that you know has good quality standards; submit subsamples at random; submit samples with known values as checks.
3. Ask for the report format and assess what needs to be done to accommodate your data management process.
4. If possible, assess the turnover of lab staff: specialized, dedicated and highly-trained staff are essential to consistently produce high quality outputs in a routine analysis laboratory.
5. Always discuss with the laboratory the purpose of the study and/or your specific requirements.

3.2.4. Definitions of protein quality in QPM

QPM is defined by the content of tryptophan and lysine. However, one of the best and widely adopted criteria for classifying a QPM cultivar is based on the protein quality index (PQI). PQI is the result of dividing the % of tryptophan by the % of protein. Normally when the PQI is higher than 0.6, the cultivar is considered QPM; however, it is also important to take into account the lysine and tryptophan contents (Table 5).

Protein and tryptophan contents in QPM grain were generally stable across environments, as determined in a study conducted at 13 tropical locations in four continents under non-limiting soil nitrogen conditions (Pixley and Bjarnason, 2002; Table 5). However, the protein, tryptophan and lysine contents in the grain endosperm of both conventional and QPM maize were found to decrease as available N in the soil decreased (Mosisa et al., 2008). A recent study found protein concentration in QPM grain to be more sensitive than tryptophan concentration to low N stress, resulting in a higher PQI under low N conditions than under optimum nitrogen supply (Wegary et al., 2011). The study also found that the percentage of tryptophan content in QPM hybrids grown under low N conditions was higher than the tryptophan content of non-QPM hybrids grown under optimal N conditions (Table 5). The stability of protein, tryptophan and lysine contents in QPM hybrids across drought-stressed and non-stressed environments was also studied, and slight increases in protein quality under stressed conditions was observed, mainly because of a concentration effect due to the small kernel size (Zaidi et al., 2008).

3.2.5. Recommendations for QPM Quality Control

Recommendations for monitoring protein quality at various stages of varietal development, as well as for quality recovery (following varietal contamination, if any), variety registration, QPM seed production, and QPM utilization and processing (including QPM in commercial products) are summarized in Table 6. The recommended laboratory quality control methodology, critical values and/or acceptable ranges are also indicated, as well as suggestions as to who should be responsible for monitoring quality at each stage.

Listed below are considerations and general recommendations to be followed when establishing and managing a QPM quality assurance system:

- Internal and external quality control units in the public and private seed industry should be strengthened to include standards and capacity for QPM quality control.
- Statutory national seed production systems and regulations (on breeder, pre-basic, basic, certified seed classes) must be respected and QPM seed production must be subjected to all established seed production rules and regulations.
- Regulatory bodies should ensure maintenance of protein quality standards in all classes of QPM seed (breeder, foundation and certified seed) as part of seed inspection and certification.
- Standard procedures for sampling certified QPM seed lots should be followed (ISTA, 2014), with sub-samples of the correct size taken appropriately, for quality analysis in an accredited or qualified laboratory.
- To safeguard protein quality and endosperm modification in QPM open-pollinated varieties (OPVs), the standard procedure for seed production (CIMMYT, 1984) may be modified at the breeder seed stage, where it is recommended to use the half-sib method in which seed samples of families planted as male rows and contributing pollen are regularly analyzed for protein quality (Vivek et al., 2008). QPM OPV seed producers should ensure that they produce foundation seed from fresh stock of breeder seed and do not exceed three cycles of planting from each new stock of breeder seed.

Table 5. Protein, lysine and tryptophan contents in whole grain samples of QPM and conventional maize genotypes produced under non-limiting nitrogen fertility in various environments.

Type of maize germplasm	Origin of samples	No. of sites	No. of samples	Kernel quality characteristics: Protein (g/100 g sample) Mean	Protein Range	Lysine (g/100 g protein) Mean	Lysine Range	Tryptophan (g/100 g protein) Mean	Tryptophan Range	Quality index 100 * %trp/ %protein Mean	Quality index Range	Source of data
CIMMYT QPM gene pools	Mexico		13	9.4	8.9-10.2	4.3	3.8-4.5	1.00	0.90-1.10	1.07*	0.94-1.21	Prasanna et al., 2001
Superior QPM hybrids in International Trials	L. America, Asia, Africa	15-23	9	-	-	-	-	0.93	0.80-1.00	-	-	Prasanna et al., 2001
Non-QPM hybrid checks in International Trials	L. America, Asia, Africa	15	2	-	-	-	-	0.65	0.60-0.70	-	-	Prasanna et al., 2001
QPM hybrids	L. America, Asia	41	8					0.90	0.86-1.0			Vasal, 2002
Non-QPM hybrid check	L. America, Asia	41	1					0.50				
CIMMYT & India QPM Inbred lines crossed in diallel	India		86	9.0	6.9-11.3							Kassahun and Prasanna, 2004
CIMMYT & India QPM Inbred lines crossed in diallel	India		50					0.95	0.60-1.18			Kassahun and Prasanna, 2004
CIMMYT QPM populations	Mexico		10	9.5	9.2-10.0	4.1	3.8-4.3	1.00	0.92-1.10	1.05	0 .93-1.18	Vasal, 2001
Released Ghanaian QPM varieties	Kumasi, Ghana		4	9.6	9.5-9.8	4.0	3.4-4.2	1.11	1.02-1.28	1.11	1.10-1.27	Ahenkora, 1999
Released Ghanaian non-QPM OPVs	Kumasi, Ghana		2	9.9	9.9-9.9	2.7	2.3-3.1	0.62	0.61-0.62	0.63	0.63	Ahenkora et al., 2000
QPM Single crosses	Kumasi, Ghana		5	8.5	8.0-8.7	-	-	0.96	0.95-0.96	1.13	1.10-1.12	Bjarnason & Vasal, 1992
30 QPM hybrids across stress and non-stress environments	India		30	9.8	±0.77	4.7	±0.06	0.89	±0.012	0.91	0.91	Zaidi et al., 2008
QPM OPV – Obatanpa	Kumasi, Ghana		1	9.8	-	-	-	0.85		0.87	0.87	Twumasi-Afriyie et al., 1999
Non-QPM OPV check –Okomasa	Kumasi, Ghana		1	10.4	-	-	-	0.46		0.44	0.44	Twumasi-Afriyie et al., 1992
QPM tested across 9 nitrogen environments	Zimbabwe, Kenya		2	7.2	6.8-7.5	2.5	2.5-2.5	0.55	0.55-0.55	0.78	0.74-0.81	Mosisa et al., 2009
Non-QPM tested across 9 nitrogen environments	Zimbabwe, Kenya		2	7.0	6.8-7.2	2.1	1.8-2.4	0.38	0.35-0.41	0.58	0.56-0.60	Mosisa et al., 2009
QPM single crosses tested under low N	Zimbabwe, Ethiopia		105	8.3	6.7-11.0	-	-	0.71	0.48-0.97	0.88	0.44-1.14	Wegary et al., 2011
Conventional hybrid check under low N	Zimbabwe, Ethiopia		2	8.0	6.6-9.4	-	-	0.54	0.53-0.55	0.68	0.59-0.77	Wegary et al., 2011
QPM single crosses tested under optimum N	Zimbabwe, Ethiopia		105	10.4	8.8-12.0	-	-	0.83	0.50-1.03	0.80	0.50-1.00	Wegary et al., 2011
Conventional hybrid check under optimum N	Zimbabwe, Ethiopia		2	11.7	10.5-12.8	-	-	0.70	0.65-0.75	0.60	0.58-0.62	Wegary et al., 2011

- Transfer of seed of any QPM germplasm (population, inbred line or hybrid) between organizations for the purpose of seed production or maintenance must be accompanied by the following information:
  1. Name of the sender
  2. Name and address of institution
  3. Name of the QPM variety/pedigree
  4. Source/origin of seed
  5. Date of production
  6. Quantity of seed
  7. Protein content (g per 100 g-sample)
  8. Tryptophan content (g per 100 g-sample)
  9. Quality Index (g-tryptophan per 100 g of protein)
  10. Authentication signature

- National Agricultural Research Systems (NARS) may seek the assistance of CIMMYT or IITA as the current major providers of QPM germplasm, to verify and ensure that QPM seed in production or proposed for release has the required genetic purity and protein quality according to the established standards. Assistance may be offered at two levels: (1) analysis of seed samples (on a cost-recovery basis) at CIMMYT or IITA laboratories to confirm the desired quality based on appropriate standards and methods; and (2) provision or verification of protein and tryptophan content in QPM reference standards used in routine analysis in the NARS QPM quality control laboratory, once established and operational. If CIMMYT is unable to provide direct assistance, it may put NARS in touch with third party analytical services (for example, HPLC amino acid profile analysis for newly released and registered varieties).

Table 6. Recommended scheme for QPM quality assurance monitoring.

		Recommended QPM quality monitoring trait					
Monitoring Stage	**Type of QPM product**	**Light table endosperm modification**	**Kernel protein**	**Kernel tryptophan**	**Kernel lysine**	**Protein quality Index**	**Who should do it**
1.0 Breeding	F2 seed	Yes	No	No	No	No	Breeder
	F3-F4 seed	Yes	Yes	Yes	No	Yes	Breeder and chemical analyst
2.0 Quality recovery following contamination, if any	F2 seed	Yes	No	No	No	No	Breeder and chemical analyst
	F3-F4 seed	Yes	Yes	Yes	No	Yes	Breeder and chemical analyst
3.0 Variety registration	Variety - Hybrid/OPV seed	Yes	Yes	Yes	Yes	Yes	Seed regulatory body
4.0 Seed bulking							
4.1 Breeder seed	Breeder seed	Yes	Yes	Yes	Yes	Yes	Seed regulatory body and breeder
4.2 Foundation: Pre-basic seed[1]	Foundation: Pre-basic seed	No	Optional	Optional	No	Optional	Seed producer and chemical analyst
4.3 Foundation: Basic seed[1]	Foundation: Basic seed	No	Optional	Optional	No	No	Seed producer and chemical analyst
4.4 Certified seed	Certified seed	No	Yes	Yes	No	No	Seed regulatory body Seed producer and chemical analyst
5.0 QPM Grain							
5.1 Farmer production	Variety - Hybrid/OPV grain	No	No	No	No	No	Farmer
5.2 Research on QPM	Variety - Hybrid/OPV grain	Yes	Yes	Yes	Optional	Yes	Researcher
5.3 Grain trading	Variety - Hybrid/OPV grain	No	Optional	Optional	No	No	Grain trader
5.4 Grain purchasing	Variety - Hybrid/OPV grain	No	Optional	Optional	No	No	Grain purchaser
6.0 QPM food/feed processing	Variety - Hybrid/OPV grain	No	Yes	Yes	Yes	No	Processor
7.0 QPM food/feed products	Flour or other QPM-labeled products	No	Yes	Yes	Yes	Yes	Processor/Bureau of Standards

[1] Monitoring QPM quality of pre-basic and basic seed is particularly desirable when these classes of seeds are produced by one company and handed over to another.

3.2.6. Maintaining nutritional quality of QPM grain and recycled seed

Because the QPM trait is conferred by a recessive gene (*o2o2*) and modifiers, the protein quality of QPM grain produced in farmers' fields as well as QPM grain (usually from OPVs) recycled as seed by farmers can depreciate over the years mainly through contamination by pollen from conventional maize grown in close vicinity, if the flowering periods overlap. Twumasi-Afriyie et al. (1996) studied contamination of a white QPM OPV by a conventional yellow maize variety planted on all sides of one-acre (0.4 ha) QPM plots at several sites in Ghana. Penetration of contaminant pollen from the plot borders inwards was estimated by examining the proportion of yellow kernels on the QPM ears; if the yellow trait was dominant, this indicated pollination by the conventional maize variety. Contamination of QPM declined to insignificant levels approximately 12 m from the nearest conventional maize plant.

Machida et al. (2012) conducted a similar study in Zimbabwe using 50 m x 42 m plots, considered representative of smallholder plot size in eastern and southern Africa. Using geostatistical analysis, they found that the percent outcrossing of QPM with conventional yellow maize fell to <20% within 5 m and <10% within 10 m of the windward plot border. Among nine different plots, the percentage of the plot area with <20% contamination was 73-92%, while the weighted average outcrossing percentage (i.e., percent contamination if grains from the entire plot were harvested and bulked) was 11.7-17.5%. A study in Ghana by Ahenkora et al. (1999) found that physical dilution of QPM grain with conventional maize grain had no significant effect on the protein efficiency ratio (PER, g weight-gain per g protein-consumed) in rats for dilutions up to 20% (Ahenkora et al., 1999). These results suggest that the levels of contamination likely to occur under on-farm QPM production conditions are not sufficient to affect grain nutritional quality in the total harvest.

Since contamination largely occurs on the perimeter of plots on the leeward side of proximal non-QPM plots, farmers can minimize contamination when there is a high probability of outcrossing by conventional maize, by adopting the following measures: (1) planting QPM in relatively square plots that minimize the perimeter length interfacing with conventional maize plots; (2) planting QPM plots upwind of conventional maize plots; and (3) harvesting and handling as non-QPM the 5 m of border rows or plants adjacent to conventional maize plots (Machida et al., 2012). Additionally, where the length of the crop season permits, farmers could also consider planting QPM varieties having different maturity, and hence, different flowering periods from conventional varieties planted in adjacent fields. Ideally, entire communities should be encouraged to adopt and plant only QPM varieties to avoid the possibility of outcrossing with conventional maize varieties. This approach was used in Ghana when QPM was first commercialized; villages were saturated with QPM seed so that virtually all maize producers in the community produced only QPM variety Obatanpa (Twumasi-Afriyie et al., 1996).

The problem of minor levels of contamination of QPM OPV grain is likely to escalate if farmers recycle QPM grain as seed for several years. In this case, farmers should be advised to select seed ears (the suggested minimum is at least 300 ears per plot) from a sub-plot area no closer than 20 m to the borders of the main production plot, and to refresh their seed from commercial sources at least after every three seasons, when conventional maize varieties are grown near the QPM field.

Because of the relative ease of maintaining seed purity of QPM hybrids in contrast to QPM open-pollinated cultivars, CIMMYT embarked on a QPM hybrid breeding program in the mid-1980s following the development of diverse QPM germplasm pools and populations (Prasanna et al., 2001). Compared to OPVs, hybrids have better kernel modification, uniformity and stability and consequently require less rigorous protein quality monitoring during seed production as long as the purity of the parental lines is assured. Moreover, QPM grain production using hybrid seed requires that farmers purchase fresh seed from seed companies each season, further assuring maintenance of protein quality.

4. Conclusion

The QPM quality protein trait and its associated nutritional benefits are conferred by the *opaque2* gene. In released QPM hybrids and open-pollinated varieties where associated characteristics such as chalky endosperm have been suppressed by modifier genes, the trait is hidden, and hence, QPM cultivars cannot be visually distinguished from conventional maize varieties. Specific procedures, usually laboratory based, are therefore required to confirm the expression of the QPM trait through elevated kernel lysine and/or tryptophan content. A quality control system should be installed to ensure that the genetic purity of QPM varieties during commercial seed multiplication. QPM grain destined for consumption should contain >0.35% lysine and >0.075% tryptophan levels in a sample of whole grain flour or >4% lysine and >0.60% tryptophan in total kernel protein. Various analytical procedures are available to determine these contents; their applicability and suitability will depend on the required accuracy, precision and cost.

Implementation of appropriate standards for QPM seed should take place within the existing seed certification system. Definitions of seed lots and procedures for sampling, sample compositing, sub-sampling and sample handling have been published by national and international (ISTA, 2014) seed standard authorities. Apart from implementing rigorous procedures for protein quality analysis, no additional procedures are required for analyzing QPM quality; working samples or sub-samples thereof need to be forwarded from the routine seed testing laboratory to the QPM quality assurance laboratory, which should be formally linked to the seed testing lab.

In principle, a special monitoring system for QPM seed and grain production should be considered as an interim measure for safeguarding the integrity of the nutritional benefits of QPM. In the long term, IF:

- protein quality is maintained at the breeder and basic seed levels;
- all the conventional seed inspection standards (isolation, processing, packaging, etc.) are respected at all seed classes; and
- regular seed quality monitoring confirms a reasonable level of QPM quality in the broader production areas;

THEN the quality of QPM seed, especially certified seed, can be assured without the need for supplementary analyses.

In principle, a quality standard for QPM grain is only necessary where the grain is traded and labeled as QPM, implying a certain quality standard. Commercial maize grain processors can ensure the quality of QPM grain by either (1) producing it themselves on their own farms or under contract where they can exercise control of production conditions including isolation of QPM production fields from conventional maize fields; or (2) conducting quality control analysis of QPM grain acquired on the open market. Although in both cases quality analysis is necessary to verify and guarantee QPM standards, the latter will likely require more intensive monitoring and a sampling protocol that is, at a minimum, based on the batch size of the processing plant. More intensive sampling and analysis are necessary if batch sizes are large, in which case, potential losses would be significant should a batch need to be condemned if it is determined to be sub-standard. Further assurance of open-market QPM grain quality may be possible through in-season field inspections and advance contract purchases.

All these measures add to the cost of the final product, which must be considered when weighing the options for procuring and producing processed QPM food products.

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Annex 1. Tryptophan and Lysine Determination Using Glyoxylic Acid

a. Tryptophan determination in maize grain

Introduction: The nutritional value of QPM is superior to that of non-QPM because it contains about double the amounts of kernel lysine and tryptophan, which are the most limiting amino acids in maize protein. Determination of tryptophan concentration is a necessary step to develop QPM, and every QPM breeding program must have access to a laboratory equipped to do tryptophan analysis. The methodology presented here is based on the method presented by Nurit et al. (2009). A high-throughput modification using a 96-well plate is presented in Galicia et al. (2012).

Principle: Tryptophan reacts with glyoxylic acid in the presence of sulfuric acid and ferric chloride, producing a colored compound that absorbs at 560 nm.

Scope: This procedure is applicable to any maize variety. Two qualified technicians can process at least 60 samples per day.

Special room conditions: None.

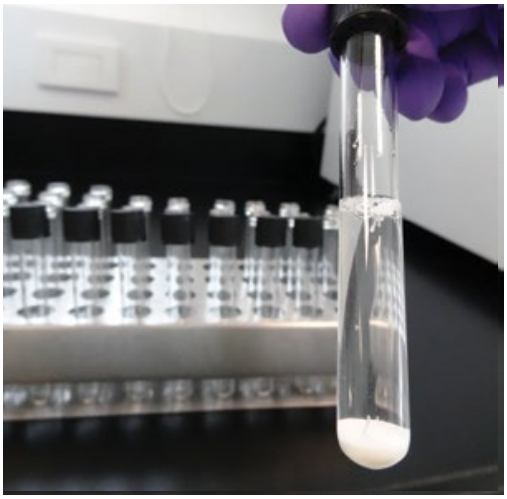
Materials:

- Analytical balance
- 10-mL glass tubes with cap
- Vortex mixer
- Incubation oven
- Spectrometer
- 1-L Erlenmeyer flask
- 5-mL pipette
- Magnetic stirrer
- Stir bar
- 500-mL volumetric flask
- 10-mL reaction tubes
- Potentiometer
- Centrifuge
- Fume hood

Procedure:

***Hydrolysis*:**

1. For each sample, label glass tubes with cap and weigh 80 mg of defatted flour maize. Defatting of maize flour is important to improve accuracy and repeatability of results. When samples are not defatted, an average of 0.8% less tryptophan is detected using this protocol. It is possible to process 40-50 samples per day, including two blanks controls, 4 checks (of known tryptophan concentration: 2 QPM, 2 normal), and the standard curve, but this depends on the technician's ability.
2. Add 3 mL of papain solution to each tube and make sure the tubes are properly closed to avoid evaporation during incubation.



Annex Figure 1. Adding papain to each tube.

3. Vortex the samples thoroughly and place them in an oven at 64°C for 16 h (overnight). If possible, vortex them twice more – one hour after being placed in the oven, and one hour before they complete the 16 h incubation time.

Annex Table 1. Reagent preparation for tryptophan determination using glyoxylic acid.

Reagent/ mixture	Specific reagents	Preparation	Special recommendations
0.1 M acetate solution $CH_3COONa•3H_2O$	Sodium acetate (JT Baker Cat. 3460 CAS 6131-90-4).	Weigh 13.53 g of sodium acetate for 1 liter of distilled water, dissolve and adjust to pH 7.0.	Stable for several weeks if stored at 4°C.
Papain solution 1 mg mL^{-1}	Papain (Mixim 0.10 MCU)	Weigh 40 mg of papain for 40 mL of solution. Dissolve the papain in the sodium acetate solution at room temperature.	Prepare it every time you use it and prepare more than you need; make sure that the phosphate buffer is at room temperature; make sure that papain powder is well dissolved.
Reagent A: 30 N sulfuric acid (Reagent C)	Sulfuric acid (JT Baker Cat. B5991-18 CAS 7664-93-9)	Place a 1-L volumetric flask on ice and add 166.7 mL of distilled water. Slowly and carefully add 833.3 mL of sulfuric acid (98%). Complete final volume with deionized water.	Prepare in a fume hood.
Reagent B: 7 N sulfuric acid	Sulfuric acid (JT Baker Cat. B5991-18 CAS 7664-93-9)	Place a 150-mL volumetric flask on ice and add at the same time 35 mL of 30 N sulfuric acid and 115 mL of distilled water. Complete final volume with distilled water.	Prepare in a fume hood.
Reagent C: 0.1 M glyoxylic acid	Glyoxylic acid (Sigma-Aldrich Cat. G10601 CAS 563-96-2)	Weigh 0.94 g of glyoxylic acid and place it in a 100-mL volumetric flask. Add 50 mL of 7 N H_2SO_4. Shake the flask until the glyoxylic acid is completely dissolved. Adjust volume to 100 mL with 7 N H_2SO_4.	Prepare it daily. Glyoxylic acid is highly hygroscopic and must be stored in a desiccator.
Reagent D: 1.8 mM iron (III) chloride	Iron (III) chloride (Sigma-Aldrich Cat. F2877-100G CAS 10025-77-1)	Dissolve 0.050 g of $FeCl_3•6H_2O$ in 100 mL of reagent C in a volumetric flask.	Prepare it daily. Be sure everything is dissolved. Be aware that $FeCl_3$ is highly hygroscopic and must be stored in a desiccator.
Reagent E: Colorimetric reagent	Reagents A and D	One hour before use, prepare a mixture of reagents A and D, ratio 1:1. Prepare the needed volume.	Prepare it daily. Protect it from light and oxygen.
Tryptophan stock solution (100 µg mL^{-1})	DL-Tryptophan (Merck Cat. 1.08375.0025)	Dissolve 10 mg of DL-Tryptophan in 100 mL of distilled water.	Prepare it weekly and store it at 4°C; vortex thoroughly before you prepare dilutions for standard curve. DL-Tryptophan must be stored in a desiccator.

4. Take the tubes out of the oven and let them cool down at room temperature (around 25 min).
5. Vortex the tubes immediately before centrifuging them at 3,000 g for 20 min. Make sure the supernatant does not have sample particles floating in it; if it does, centrifuge again.

Annex Figure 2. Centrifugation.

Standard curve

6. Prepare a stock solution of 100 µg mL^{-1} tryptophan in deionized water: weigh 50 mg of DL- tryptophan and place it in a one-liter Erlenmeyer flask. Add 250 mL of deionized water and a stir bar. Place the flask in a magnetic stirrer to dissolve the tryptophan; this takes around 1 hour.
7. Transfer the solution to a 500-mL volumetric flask and proceed to dilute to the mark with the diluent.
8. In 10 ml reaction tubes, prepare daily 0, 10, 15, 20, 25 and 30 µg mL^{-1} dilutions (see Table 1). Vortex properly before further use.

Colorimetric reaction

9. Transfer 1 mL of hydrolysate (supernatant) of each sample to the 10-mL glass tubes.
10. Add 3 mL of reagent E (colorimetric reagent) to each tube.
11. Close the tubes and shake in a vortex.
12. Incubate the tubes at 64°C for 30 min for color development.
13. Take the samples out of the oven and let them cool down at room temperature for 25 min.
14. Read absorbance in the spectrometer at 560 nm.

Calculations

15. The equation used to determine the tryptophan percentage in each sample is:

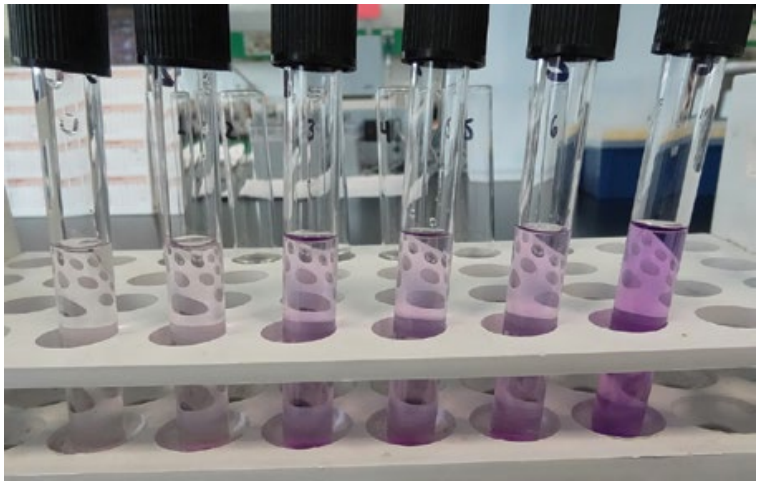
$$\%trp = \frac{ODcorr^{560nm}}{slope} \times \frac{Hydrolysate\ volume\ (mL)}{sample\ weight\ (\mu g)} \times 100$$

Where:

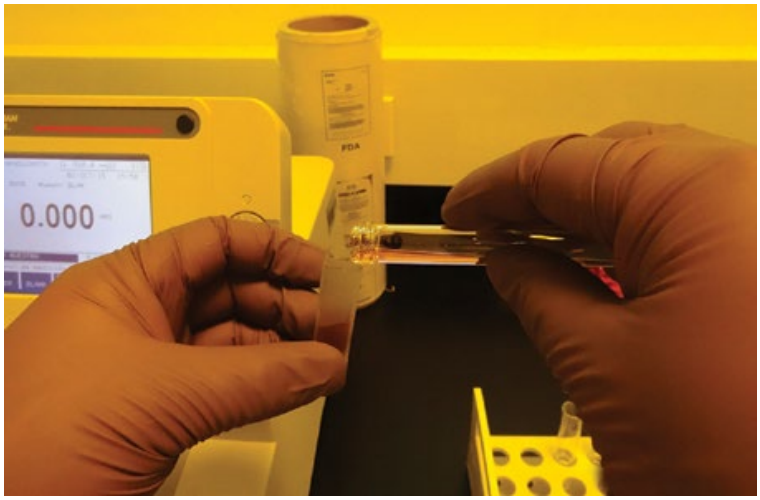
- ODcorr560nm = Corrected optical density (absorbance) at 560 nm. It is called “corrected” because it is the OD of each sample minus the average OD of the blanks. Papain is a protein that contains a large quantity of tryptophan (each papain molecule contains 7 trp units), so it is necessary subtract this quantity to do the calculations.
- Hydrolysate volume: 1.125 mL in this protocol.
- Sample weight: 80 000 µg.
- The slope is calculated from the standard curve, where the independent variable is the tryptophan concentration and the dependent variable is the absorbance.

Annex Table 2. Preparation of the tryptophan standard.

Tube Nº	Stock trp µg mL^{-1} (mL)	Deionized water (mL)	Total volume (mL)	Trp concentration [µg mL^{-1}]
1	0.0	5.0	5.0	0.0
2	0.5	4.5	5.0	10.0
3	0.75	4.25	5.0	15.0
4	1.00	4.0	5.0	20.0
5	1.25	3.75	5.0	25.0
6	1.50	3.5	5.0	30.0



Annex Figure 3. Colorimetric reaction in standard curve.



Annex Figure 4. Reading absorbance.

Annex Table 3. Trouble-shooting.

Problem	Solution
No color development in the reaction	Test another batch of colorimetric reagent. Verify quality of reagents. Verify temperature in the incubation oven.
The OD for the papain blank is too high or too low (less than 0.035 or more than 0.050).	Verify that the amount of papain is correct. Use another batch of papain.
Low values of control samples	Make sure digestion of samples is done properly: Be sure that after sample digestion there are no particles on the tube wall. If there are, vortex the sample and centrifuge again for 15 min. Verify incubation was done at 64 °C for 16 hours. Verify quality and quantity of the reagents used. Verify quality of tryptophan standard curve: Be sure that the stock solution of tryptophan is properly dissolved before you do the dilutions. Mix the stock solution of tryptophan very well before you do the dilutions. Prepare new stock solution of tryptophan.
OD measurements between replicates vary too much	Verify the accuracy of the sample weights. Make sure replicates are analyzed in the same way, using the same batch of reagents. Make sure the samples have cooled to room temperature before reading. Set the spectrophotometer to"zero" again and be sure it is stable before you read your sample.
Papain does not dissolve	Make sure the acetate solution is at room temperature.

B. Lysine determination in maize grain

Introduction: Lysine is an essential amino acid, which means it is necessary for human health; the problem is the body cannot make it, so it has to obtain lysine from food. Maize has low lysine content but is the principal energy source in the diet of people in many different countries. Therefore need to have maize with high quantity of lysine and the improvement are working in this topic.

Principle: The colorimetric procedure for lysine quantification is based on two steps. The first step is the protection of the amino group in ∝ of the lysine chain by reaction with copper; copper also blocks the amino group of low molecular weight peptides presents in the hydrolysate. The second step is the reaction of the 2-chloro-3-5-dinitropyridine with the amino group in ξ of the lysine chain to give a colored ξ-dinitropyridil lysine which is determined spectroscopically at 390 nm.

Scope: This procedure is applicable to any maize variety. Two qualified technicians can process up to 80 samples per day.

Special room conditions: None.

Materials:

- Analytical balance
- 5 mL Falcon tubes with cap
- Vortex mixer
- Incubation oven
- Spectrometer
- 1 L glass flask
- 200, 1000 and 5000 µL micropipettes
- Potentiometer
- Centrifuge
- Fume hood
- Magnetic stirrer
- Stir bar
- 500 mL volumetric flask
- 5 mL reaction tubes
- 10 mL reaction tubes
- 20 mL reaction tubes

Annex Table 4. Reagent preparation for lysine determination.

Reagent/ mixture	Specific reagents	Preparation	Special recommendations
0.03 M phosphate buffer solution, pH 7.4	Sodium phosphate dibasic Na_2HPO_4 (JT Baker Cat. 3828 CAS 7558-79-4). Potassium phosphate monobasic KH_2PO_4 (JT Baker Cat. 3246).	Dissolve 3.19 g of sodium phosphate in 400 mL distilled water; dissolve 1.04 g of potassium phosphate in 300 mL distilled water; mix both solutions and complete volume to 1 L with distilled water; adjust to pH 9.0.	Stable for several weeks if stored at 4 °C.
Papain solution 4 mg mL^{-1}	Papain (Mixim 0.10 MCU)	Dissolve 800 mg of papain in 200 mL of 0.03 M phosphate buffer solution (always prepare it fresh and at least 50 mL more than you need).	Prepare it every time you use it; make sure the phosphate buffer is at room temperature; make sure the papain powder is well dissolved.
Papain solution 5 mg mL^{-1}	Papain (Mixim 0.10 MCU)	Dissolve 125 mg of papain in 25 mL of 0.03 M phosphate buffer solution.	Prepare it every time you use it; do not store the solution; prepare just the necessary volume.
0.6 M carbonate buffer solution, pH 9.0	Sodium carbonate Na_2CO_3 (JT Baker Cat. 3602); sodium bicarbonate $NaHCO_3$ (JT Baker Cat. 3605-05).	Dissolve 6.36 g of sodium carbonate in 100 mL distilled water; dissolve 25.2 g of sodium bicarbonate in 500 mL distilled water; mix both solutions and adjust pH to 9.0.	Store at 4 °C.
0.05 M borate buffer solution, pH 9.0	Sodium borate decahydrate; $Na_2B_4O_7.10H_2O$ (Sigma Aldrich Cat. S-9640).	Dissolve 19.07 g of sodium borate in 1000 mL distilled water; adjust pH to 9.0.	Store at 4 °C.
Copper phosphate suspension	Copper (II) chloride dehydrate; $CuCl_2.H_2O$ (JT Baker Cat. 1792-01); sodium phosphate tribasic dodecahydrate ($Na_3PO_4.12H_2O$(JT Baker Cat. 3836)	Dissolve 2.8 g of cupric chloride in 100 mL deionized water; dissolve 13.6 g of sodium phosphate tribasic in 200 mL deionized water; mix both solutions and shake for 1 hour; divide the volume into 8 centrifuge tubes (approx. 37.5 mL per tube); centrifuge at 2500 rpm for 5 min; remove the supernatant and re-suspend the pellet three times in 15 mL of borate buffer solution, removing the supernatant. Re-suspend the pellet in 10 mL of borate buffer solution, mix well and keep the solution.	Store at 4 °C.
1.2 N hydrochloric acid	Hydrochloric acid HCl (Merck Cat. 1.00317.2500)	To prepare 1000 mL: Add 600 mL of distilled water to a 1-L measuring flask. Slowly and carefully add 172 mL of HCl. When the solution is at room temperature, complete the volume to 1 L.	Prepare this solution in a fume hood. Stable for several weeks; store at room temperature.
Ethyl acetate	Ethyl acetate 99.9% (JT Baker Cat. 9280)	Use directly.	
2-chloro-3,5-dinitropyridine reagent (3% in methanol)	2-chloro-3,5-dinitropyridine (Merck Cat. 8141340001). Methanol (JT Baker Cat. 9093-03).	Dissolve 240 mg of 2-chloro-3,5-dinitropyridine in 8 mL of methanol.	Prepare it daily, as needed; protect from light and oxygen; be sure that dinitropyridine is dissolved completely.
Amino acid mixture	DL-amino acids (Sigma Aldrich Cat. 6364).	Weight: 20 mg each of: cystine, methionine, proline; 30 mg each of: histidine, alanine, isoleucine, threonine, tyrosine; 40 mg each of: glycine, phenylalanine, valine; 50 mg each of: arginine, serine; 60 mg of aspartic acid; 80 mg of leucine; 300 mg of glutamic acid. Mix all amino acid powders; weigh 100 mg and dissolve in 10 mL of carbonate buffer solution.	Store the mixed powder at 4 °C; do not store the solution.
Lysine stock solution (1000 µg mL^{-1})	L-Lysine monochloride (Nutritional Biochemical Corp.)	Dissolve 10 mg of DL-Tryptophan in 100 mL of deionized water.	Prepare in a fume hood.

Procedure:

***Hydrolysis*:**

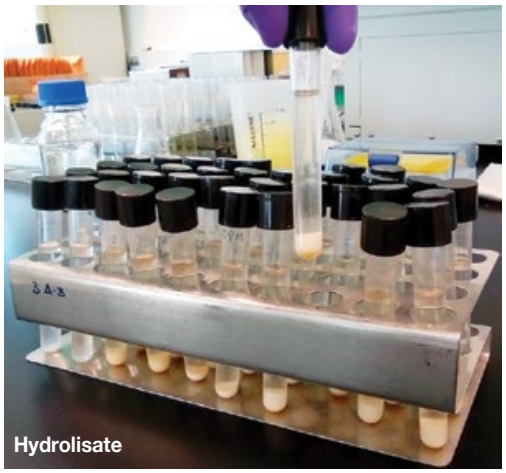
16. For each sample, label falcon tubes and weigh 100 mg of defatted flour maize. Defatting of maize flour is important to improve accuracy and repeatability of results. Always include two blank controls, 4 checks (of known lysine concentration: 2 QPM, 2 normal), and the standard curve.
17. Add 5 mL of papain solution (4 mg mL^{-1}) to each tube and make sure the tubes are properly closed to avoid evaporation during incubation.
18. Vortex the samples thoroughly and place them in an oven at 64 °C for 16 h (overnight). If possible, vortex them twice more - one hour after being placed in the oven, and one hour before they complete the 16 h incubation time.
19. Take the tubes out of the oven and let them cool down at room temperature (around 25 min).
20. Vortex the tubes immediately before centrifuging them at 2500 rpm for 5 min. Make sure the supernatant does not have sample particles floating in it; if it does, centrifuge again.

Standard curve

21. Prepare a stock solution of 1000 mg mL^{-1} lysine in carbonate buffer solution.
22. In 10-mL reaction tubes, prepare daily 0, 50, 100, 150, and 200 µg mL^{-1} dilutions (see Annex Table 5); vortex properly before further use.
23. Take 1 mL of each dilution and transfer to the new tube. Add 0.5 mL of the amino acid mixture solution and 0.5 mL of copper solution and follow steps 10 to 17.

Colorimetric reaction

24. In 5-mL reaction tubes, add in order: 0.5 mL of carbonate buffer solution, 1 mL of supernatant of each sample, and 0.5 mL of copper phosphate suspension.
25. Shake manually and vigorously for 5 min. Centrifuge at 2500 rpm for 5 min.



Annex Figure 5. Hydrolysate and carbonate solution addition.

Annex Table 5. Preparation of lysine standard.

Tube N°	Stock lys µg mL^{-1} (mL)	Carbonate solution (mL)	Papain solution 5 mg mL^{-1} (mL)	Total volume (mL)	Lys concentration [µg mL^{-1}]
1	0.0	2.0	8	5.0	0
2	0.5	1.5	8	5.0	50
3	1.0	1.0	8	5.0	100
4	1.5	0.5	8	5.0	150
5	2.0	0.0	8	5.0	200

26. In 20-mL reaction tubes, transfer 1 mL of supernatant and add 0.1 mL of 2-chloro-3,5-dinitropyridine reagent and shake manually and thoroughly.
27. Keep the tubes in darkness at room temperature for 2 h, shaking after each 30 min.
28. Add 5 mL 1.2 N HCl to each tube and vortex.
29. Add 5 mL of ethyl acetate.
30. Cover the tubes; mix the solution by inverting the tubes 10 times.
31. Remove the supernatant using a micropipette. Repeat this step three times.
32. Read absorbance at 390 nm using a spectrophotometer.

Calculations:

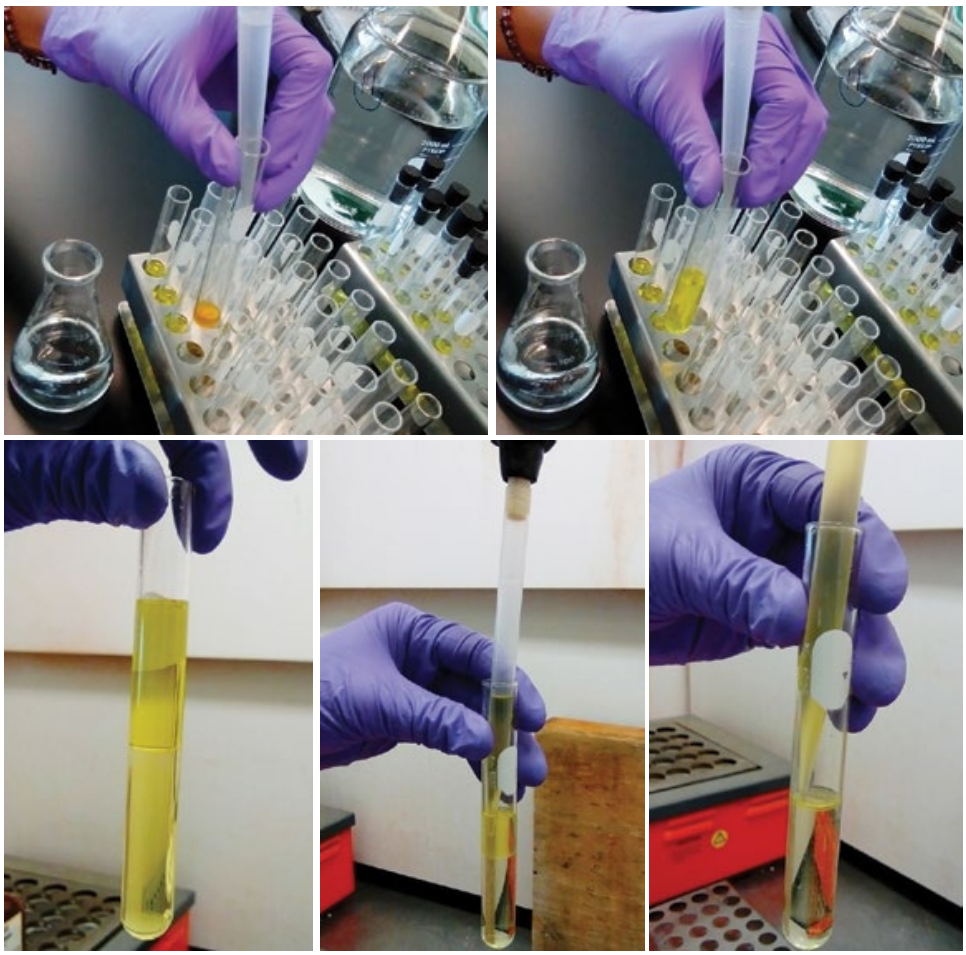
33. The equation used to determine lysine percentage in each sample is:

$$\%lys = \frac{ODcorr^{390nm}}{slope} \times \frac{Hydrolysate\ volume\ (mL)}{sample\ weight\ (\mu g)} \times 100$$

Where:

- ODcorr390nm = Corrected optical density (absorbance) at 560 nm. It is called “corrected” because it is the OD of each sample minus the average OD of the blanks.
- Hydrolysate volume: 5 mL in this protocol.
- Sample weight: 100 000 µg.
- The slope is calculated from the standard curve, where the independent variable is the lysine concentration and the dependent variable is the absorbance.

Annex Figure 6. Colorimetric reaction.



Annex Figure 7. Ethyl acetate washing.

Trouble-shooting:

Problem	Solution
No color development in the reaction	Test another batch of colorimetric reagent. Verify quality of the reagents. Verify temperature in the incubation oven.
OD for the papain blank is too high or too low (less than 0.035 or more than 0.050).	Verify that the amount of papain is correct. Use another batch of papain.
Low values of control samples	Make sure digestion of samples is done properly: Be sure that after sample digestion there are no particles on the tube wall. If so, vortex the sample and centrifuge again for 15 min. Verify incubation was done at 64°C for 16 h. Verify quality and quantity of the reagents used. Verify quality of your tryptophan standard curve: Be sure that the stock solution of tryptophan is properly dissolved before you do the dilutions. Mix the stock solution of tryptophan well before you do the dilutions. Prepare a new stock solution of tryptophan.
OD measurements between replicates vary too much	Verify the accuracy of the sample weights. Make sure the replicates are analyzed in the same way, using the same batch of reagents. Make sure samples have cooled down to room temperature before reading. Set the spectrophotometer to“zero” again and be sure it is stable before you read your sample.
Papain does not dissolve	Make sure the acetate solution is at room temperature.

Annex 2. Near-Infrared Spectroscopy for estimation of tryptophan and lysine in maize flour

Introduction

Near-infrared reflectance spectroscopy (NIRS) is a technique that combines spectroscopy and mathematics to rapidly produce indirect quantitative estimates of concentrations of OH-, NH-, CH-, or SH-containing compounds. An infrared spectrum is commonly obtained by passing infrared radiation through a sample and determining what fraction of the incident radiation is absorbed at a particular energy. The energy at which any peak in an absorption spectrum appears is the frequency of a vibration of a part of a sample molecule. The vibrations of molecules are crucial for interpreting infrared spectra.

Qualitative and quantitative NIR spectroscopic methods require the application of multivariate calibration algorithms (commonly referred to as chemometric methods) to model the spectral response to chemical or physical properties of a calibration set. The identification of unique wavelength regions where changes in the response of the NIR spectrometer are proportional to changes in the concentration of chemical components or in the physical characteristics of samples being analyzed is required for scientifically understanding the cause (i.e., molecular or physical properties) and effect (i.e., spectroscopic changes), even for routine method development. This correlation or interpretation of spectra converts the abstract absorption data (spectrum) into structural information representing the molecular details of a measured sample. Interpretive spectroscopy of this sort provides a basis for establishing known cause-and-effect relationships between the spectrometer response (spectrum) and the molecular properties of the sample.

Scope:

Near-infrared spectroscopy (NIRS) is an option in the Maize Nutritional Quality Laboratory (MNQ Laboratory) currently used for establishing the biochemical and chemical platform for analysis to support the breeding programs.

The main advantages of this technique are that it:

- facilitates sample preparation.
- is rapid and inexpensive.
- facilitates analyzing several traits simultaneously.
- is non-destructive.

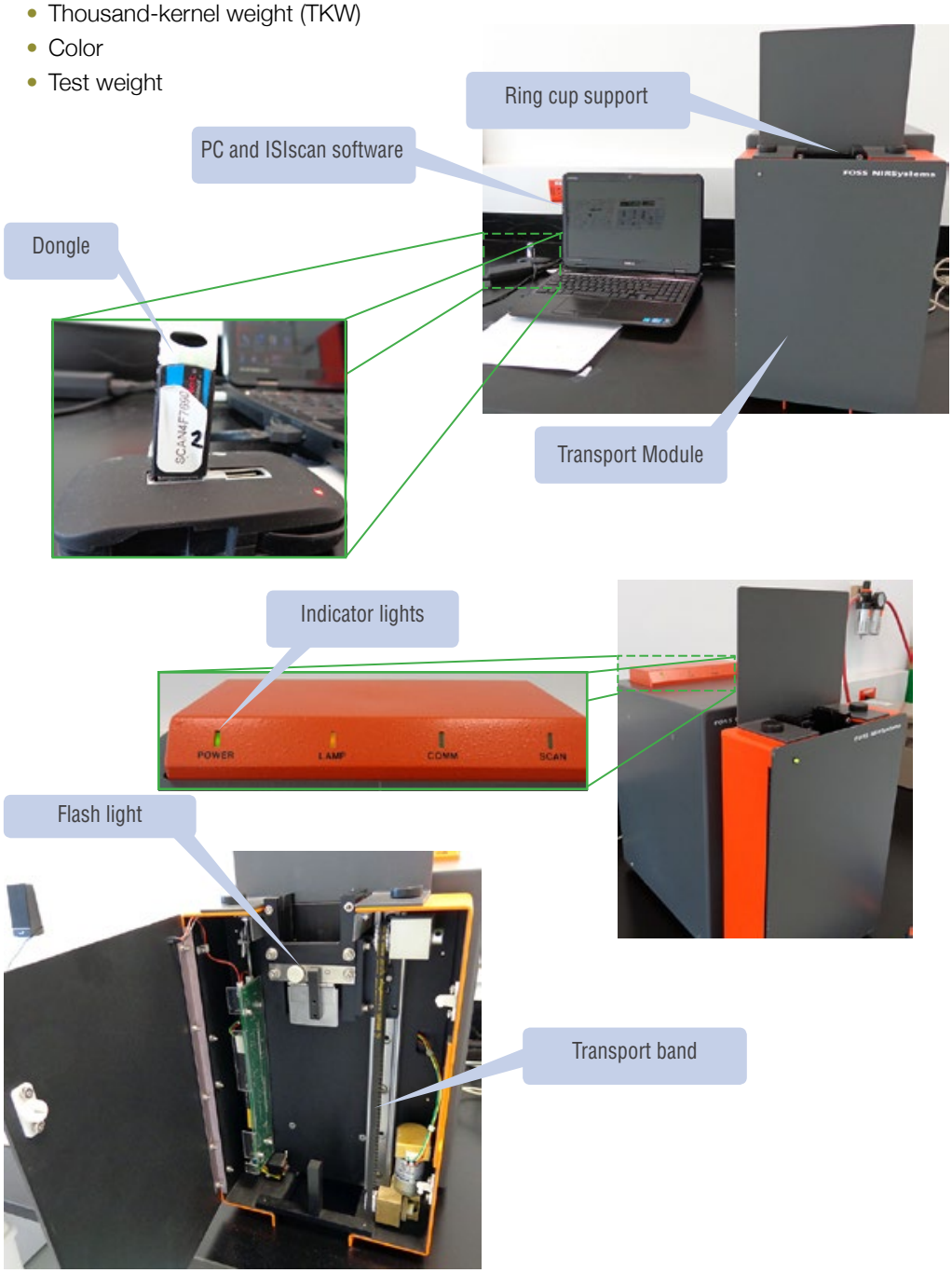
Points to consider:

- Models are based on predictions and not as accurate as wet chemistry analysis.
- It is important to regularly analyze small sets of samples by wet chemistry.
- Models are developed with a wide range of values. Samples with values outside the models have to be analyzed by wet chemistry.
- Small calibration sample sizes can lead to mis-estimations.
- Although transfer calibration between different instruments is possible, it is not a straightforward exercise. Whenever possible, it is better to develop the calibration for each instrument.
- Instruments and software are costly.

In the MNQ Lab, the NIRS equipment has calibrations to quantify the following compounds:

- In maize flour:
  - Protein
  - Tryptophan
  - Lysine
- In maize grain:
  - Protein
  - Thousand-kernel weight (TKW)
  - Color
  - Test weight
- In maize vegetable tissue:
  - Ash
  - Fiber (IVTD, NDF, ADF, ADL)

The software used is ISI scan, ver. 4.5.0.14017.



Annex Figure 8. Sample preparation.

General instrument description

Standard procedures are crucial to the successful use of NIRS for analysis. Since NIRS is responsive to both chemical and physical properties of the sample, spectra will vary depending on which method is used for sample preparation (sample drying, sample milling).

Green tissue: Green tissue is very hygroscopic so samples should be dried in an oven at 70°C for at least 6 h. This temperature is high enough that most of the water will be driven out and low enough that sample chemistry is not significantly altered. After drying, the samples should be allowed to reach room temperature (22 ± 3°C) for 30 minutes.

Maize flour: Samples should be ground with a cyclonic mill fitted with a 0.5 mm sieve. Several studies have demonstrated that the precision of the analysis is greater if samples are ground in a cyclonic mill than in another kind of mill because of the increased uniformity of particle size. The mill should be cleaned perfectly between samples to minimize cross-contamination. Sample moisture is usually around 10%. It is very important to ensure minimum variation in moisture content among samples (maximum: 3% variation). Avoid using defatted samples because the integrity of the compounds is very important to obtain a good prediction model.

We recommend using fresh samples to make prediction models. However, if you have to store the samples, put them in a sealed glass or plastic container to prevent moisture changes. Ambient room conditions are a good storage option (for 2 weeks maximum) but only if the compounds to be stored (such as carotenoids) are not sensitive to light or temperature.

NIRS lab ambient conditions

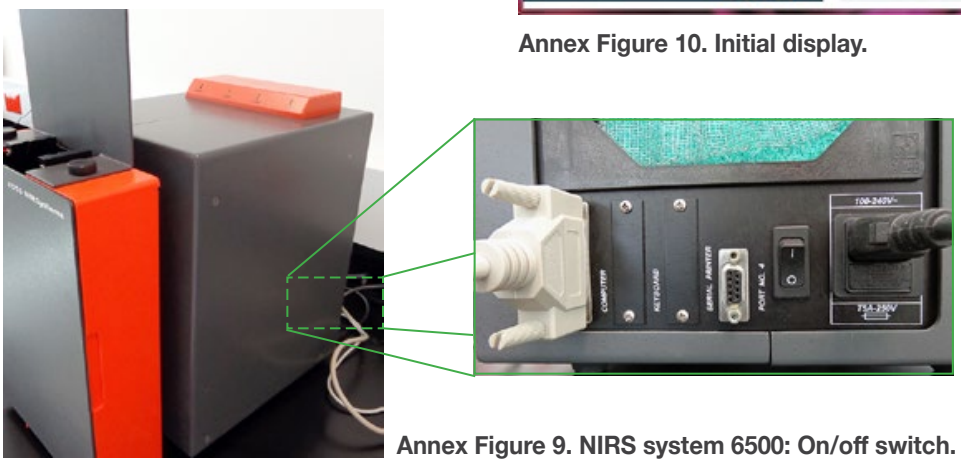
NIRS measurements are sensitive to the temperature of the instrument and the sample. Therefore, all instruments have controls that regulate internal temperature. These controls work well within a 25 ±5°C temperature range. However, the temperature of the sample is not regulated by the instrument, so the operator must make sure the samples are at constant room temperature before they are analyzed.

Ambient moisture should also be constant to avoid changes in the spectrum.

Procedure

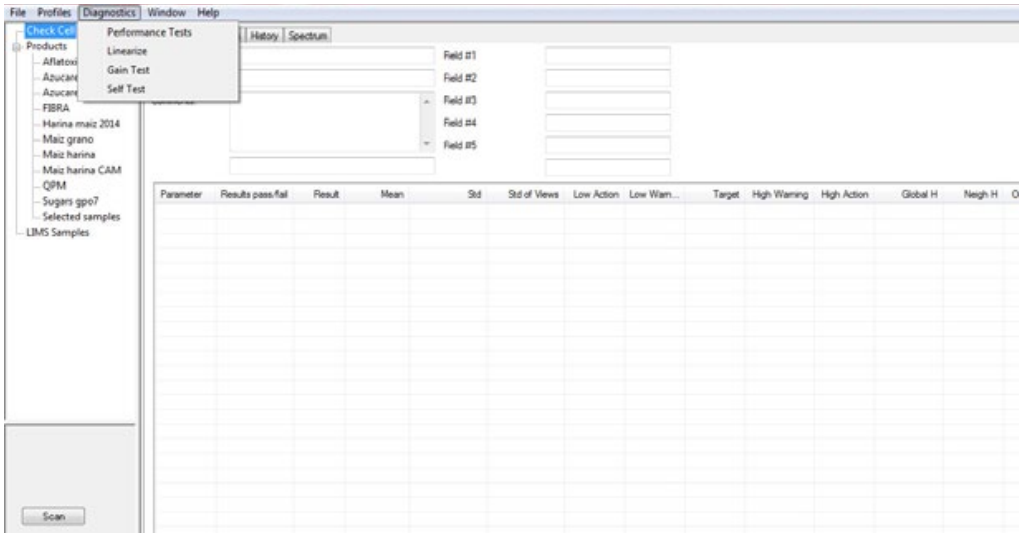
1. Turn the instrument on; the on/off switch is at the back of the instrument (Annex Figure 9).
2. When the instrument is switched on, the POWER indicator light also comes on (see General Instrument Description). Turn on the computer and open the ISIscan software.
3. Select the user name and type the password (Annex Figure 10).

Annex Figure 10. Initial display.



Annex Figure 9. NIRS system 6500: On/off switch.

4. The display will show the analysis window (Annex Figure 11). Before starting, instrument operation must be monitored using the system performance diagnostics (see Diagnostics).
5. Once diagnosis is completed, scan the check cell (Annex Figure 12). The check cell is part of routine diagnostics and consists of a feed concentrate sample cell and a specially created equation. The check cell should be analyzed every day or at the beginning of each shift. The purpose of the check cell is to show that the instrument always predicts the same values. To scan the check cell, click on CHECK CELL product and then on SCAN. The values displayed should be similar to the label values of the check cell (Annex Figure 13).
6. In the MNQ lab, the NIRS equipment is configured with different PRODUCTS (Annex Figure 14), each one with particular characteristics (type of cup, number of scans, prediction equation, etc.; for further information, consult the ISIscan User's Guide). Routine analyses are:
   a. Maiz harina (maize flour)
   b. Maiz grano (maize grain)
   c. FIBRA (maize tissue)
   d. Azucares trigo (wheat tissue)



Annex Figure 11. Diagnostics.

Annex Figure 12. Check cell: (a) container box; (b) rear view; (c) frontal view; (d) in the inbox.

Usually, the cups that contain the samples are:

- For plant tissue and grain: Rectangular cup ¼.
- For maize flour: Ring cup.

The minimum sample weight for both cups is 1 g, but the optimal weight is 2–4 g for vegetable tissue and 4–6 g for grain and flour. The amount of the sample is notvery important if it is enough to cover the cup. However, the use of consistent weights would help to develop better calibrations and obtain consistent results in the routine analysis.

7. To analyze the sample, fill the correct cell (ring cup or rectangular cup) and place it in the transport module.

   The cell must be perfectly clean. Use compressed air to clean the cell between readings. If necessary, further cleaning can be done with disposable wipes (Kimwipes). Always wear gloves when handling cells (Annex Figure 16). Avoid using solvents or soap and water to clean the cups.

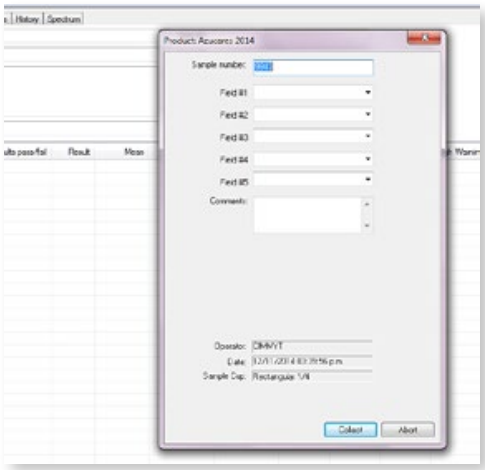
8. Start scanning the sample. Choose any of the options below:

   a. Double click on the product you wish to scan with, or

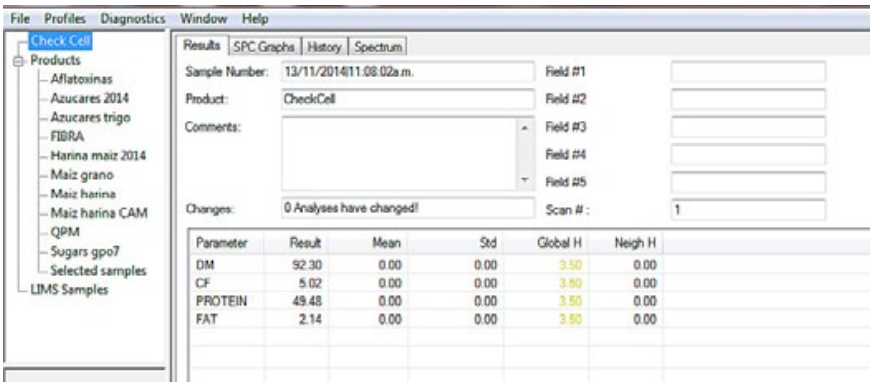
   b. Right mouse click on the product and select SCAN from the menu, or

   c. Click to select the product and then SCAN from the control window.

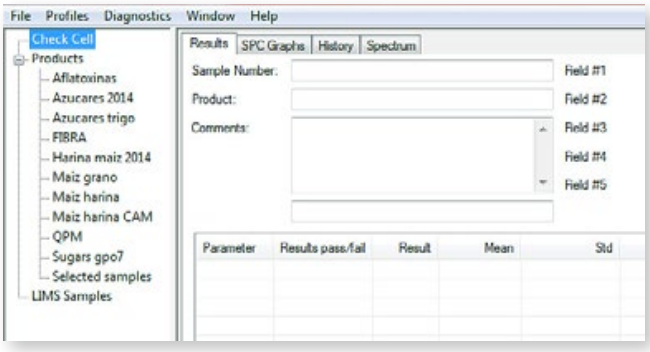
A window like the one below will appear:



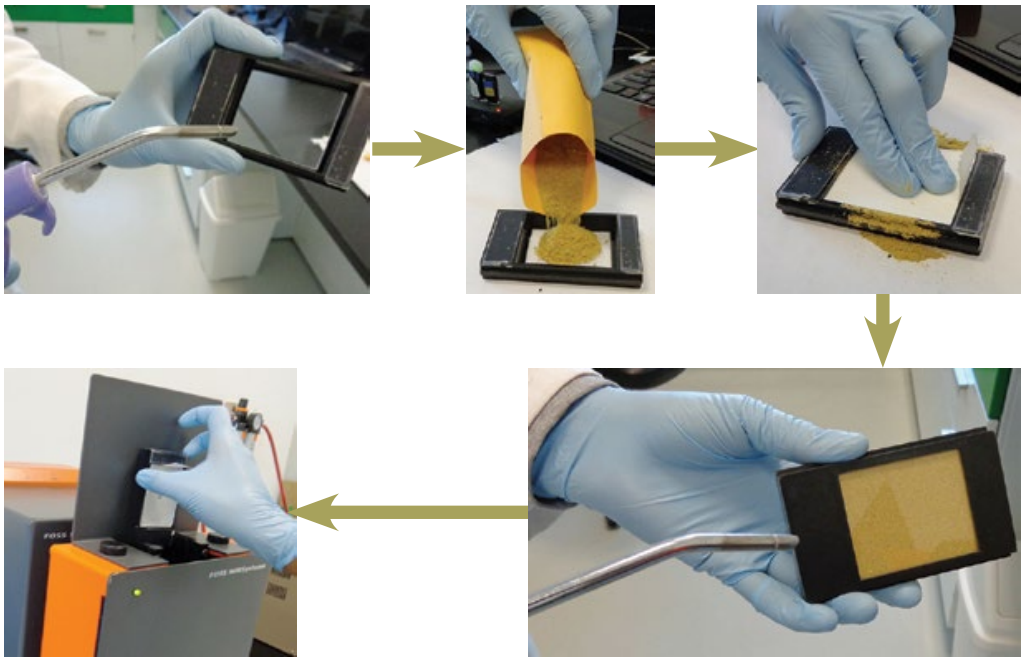
Annex Figure 15. ID sample.



Annex Figure 13. Check cell values.

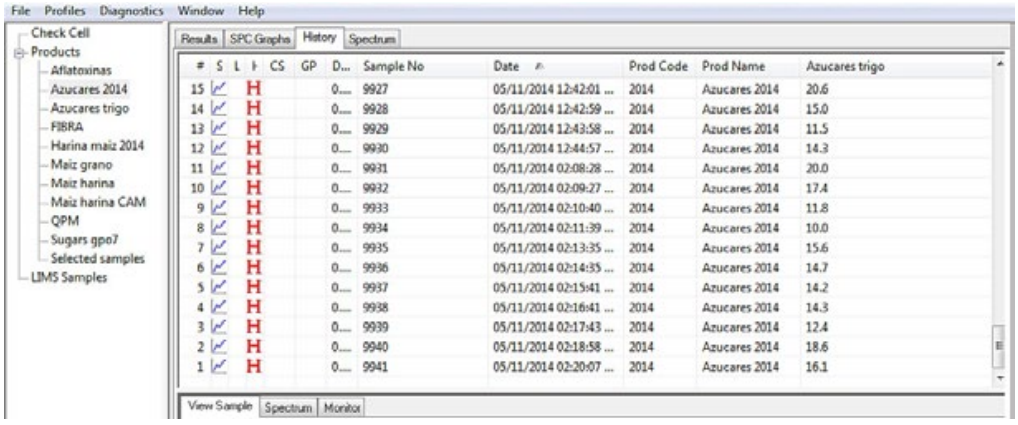


Annex Figure 14. List of available products.



Annex Figure 16. Cleaning and filling the sample cell.

9. Enter the number and description of the sample. Make sure that the sample is in the instrument and then press COLLECT. After scanning is complete, the results will be displayed. The information is automatically saved (click on HISTORY to check the values; Annex Figure 17). A new window for scanning the next sample will appear.
10. The results can be exported to an Excel file or a spectra file to be analyzed in the Win ISI software (see Export the results).
11. You can transfer the sample to the original container and use it for further analysis.
12. To switch off the equipment, close the ISI scan software. A window will appear asking if you want to switch off the lamp; click on YES. Switch off the instrument when the fan turns off (you'll know because the fan is noisy when it is working).



Annex Figure 17. History.

Diagnostics

a. *Advanced diagnostics.* Used to set up the equipment after a module or a lamp is changed, or after the instrument is repaired.

   ✓ Wavelength linearization.

   ✓ Gain test.

   ✓ Self-test.

b. *Routine diagnostics.* Should be performed following a consistent diagnostic schedule; the first indication of a possible malfunction of the instrument:

   ✓ Performance test.

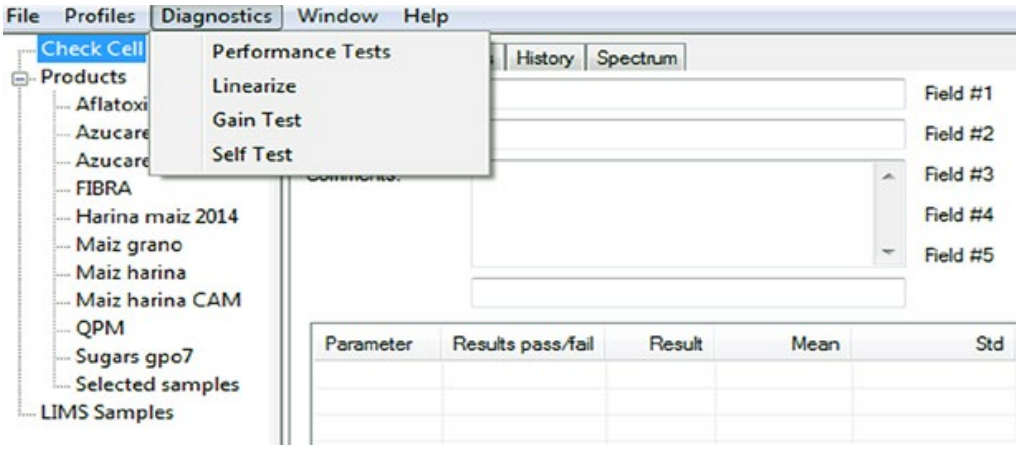
   ✓ Check cell.

We suggest performing the diagnostics in the following order:

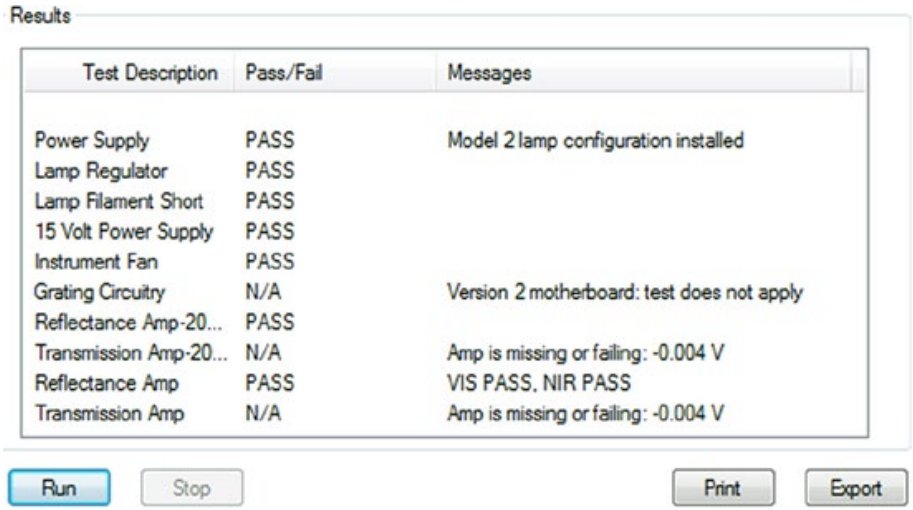
Click on DIAGNOSTICS to display the diagnostics options (Annex Figure 18).

Self-test: Used to review the instrument's motherboard and electrical circuits. This is a general test for the electronics component and can be used to troubleshoot potential instrument problems. The results are displayed when the test is finished (Annex Figure 19).

The test must indicate PASS for each part of the instrument. If an item is labeled as FAIL, the test must be repeated. If the result is still FAIL, please contact your local FOSS service technician. If FAIL refers to the INSTRUMENT FAN, please make sure the instrument has been on for at least one hour.



Annex Figure 18. Diagnostic menu.



Annex Figure 19. Results of the SELF TEST.

Gain test: This test measures the detector's response to the light source and can easily detect a bad lamp or lamp regulator. Over time, this test can also indicate a failing detector response. The test should be performed in two regions: visible (Annex Figure 20a) and infrared (Figure 19).

To start the test, select GAIN TEST in the DIAGNOSTICS menu. The gain settings and detector voltages will be displayed in the center table, along with the detector voltage on the sliding graph.

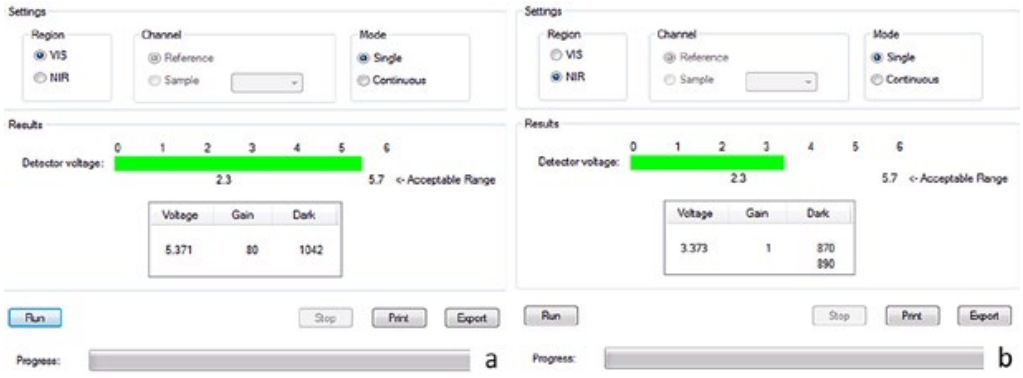
The voltage will be displayed on the bar graph. It should be between 2.3 and 5.7 volts (green bar when ok and red when it fails). The voltage is also displayed in the chart below. If the voltage is not within the acceptable range, please run the instrument self-test. The lamp may need to be replaced.

The gain in the NIR detector amplifier will typically be 1–10, while the gain in the visible detector will often be over 80. This is normal and just a function of the different detector types.

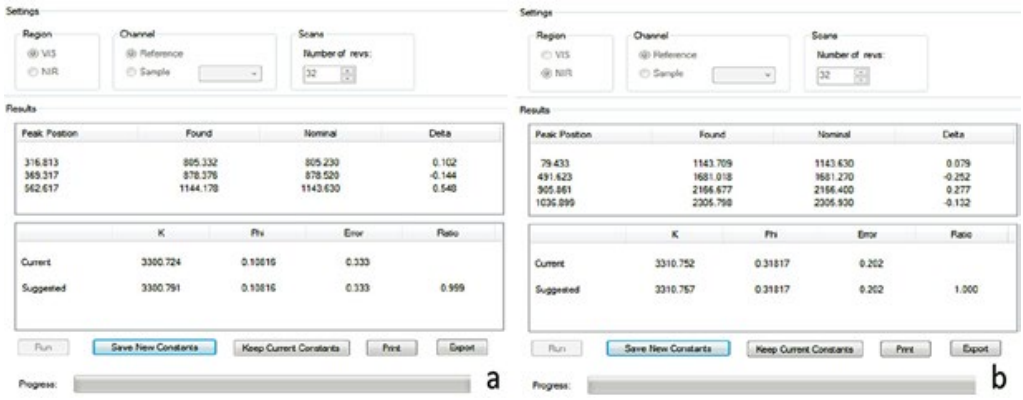
Linearize (wavelength linearization): This is a diagnostic tool that uses internal wavelength spectroscopic standards (polystyrene: NIR region; didymium: visible region) to align the monochromator. Proper alignment produces accurate predictions and consistent calibration transfers.

To start wavelength linearization, select DIAGNOSTICS and click on LINEARIZE (Annex Figure 21). The number of revolutions is the number of subscans that are averaged together for linearization. The default value is 32 and should be used unless a quick test is required during service. Select the VIS region and RUN the test (Annex Figure 21a). Upon completion, linearization data will be displayed in the tables.

To evaluate the linearization, find the Ratio field in the table below (Annex Figure 21b). This value reflects the ratio of the wavelength



Annex Figure 20. Gain test results. (a): visible; (b) NIR.



Annex Figure 21. Linearization results.

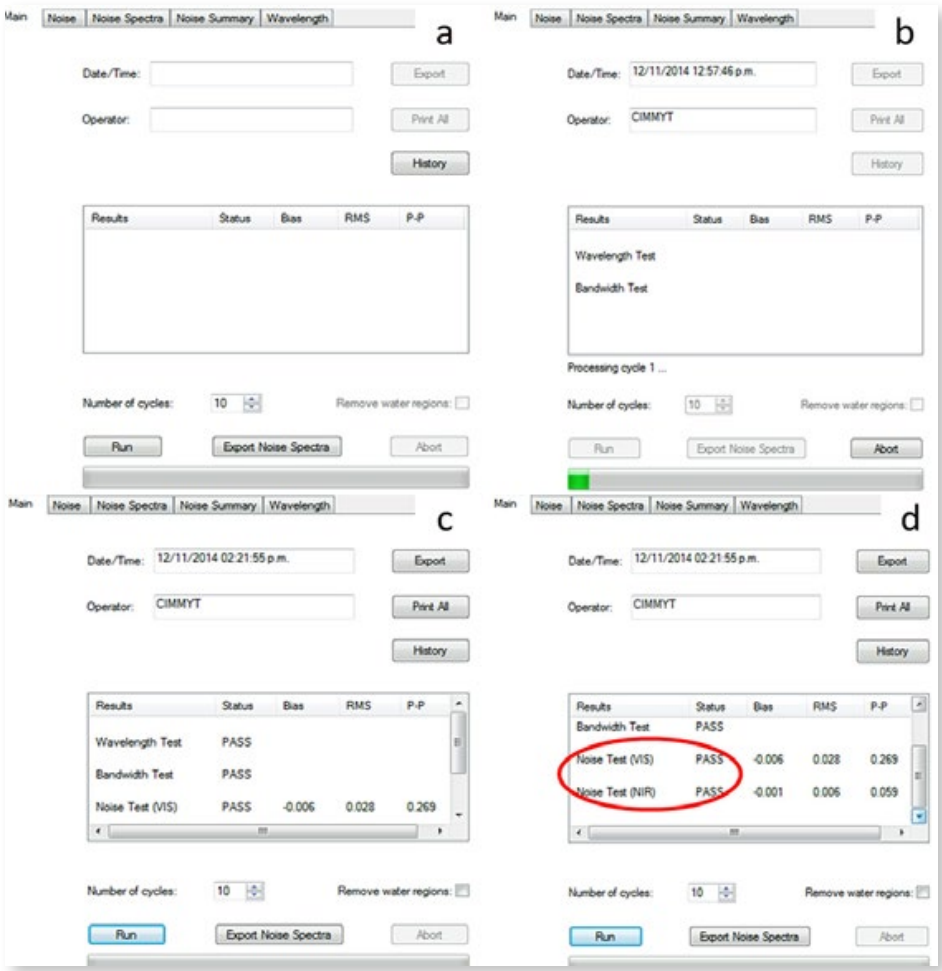
errors of the suggested settings versus the current settings. If the ratio is 0.9 or less, click on save new constants and rerun the test; if the ratio is over 0.9, select keep current constants. We recommend using a ratio of 1.0 ± 0.001. Repeat the test, but this time select the NIR region.

Performance test: The performance test is a very thorough and robust measure of instrument performance. It combines a wavelength accuracy test, bandwidth precision and accuracy tests, and a noise test. All of these are run and reported automatically during the performance test.

It is recommended that the performance test be run at least once a week. Preferably, this test should be run daily or at the beginning of each shift. Please make sure that the instrument has been initialized and warmed up for at least one hour; this will stabilize the instrument and give the most accurate diagnostics.

To start the performance test, select DIAGNOSTIC and click on PERFORMANCE TEST. A Performance test dialog will appear on the right side of the user interface, as shown in Annex Figure 22. Select RUN to initiate the test. It is very important to run this test and put a cup in the transport module because the external light could interfere and produce "noise" in the spectra.

After approximately 10 minutes, results will appear in the results window. The Wavelength test, Bandwidth test and Noise test should all show PASS. If any of these tests do not pass, allow the instrument to warm up another 15 minutes and repeat the test.



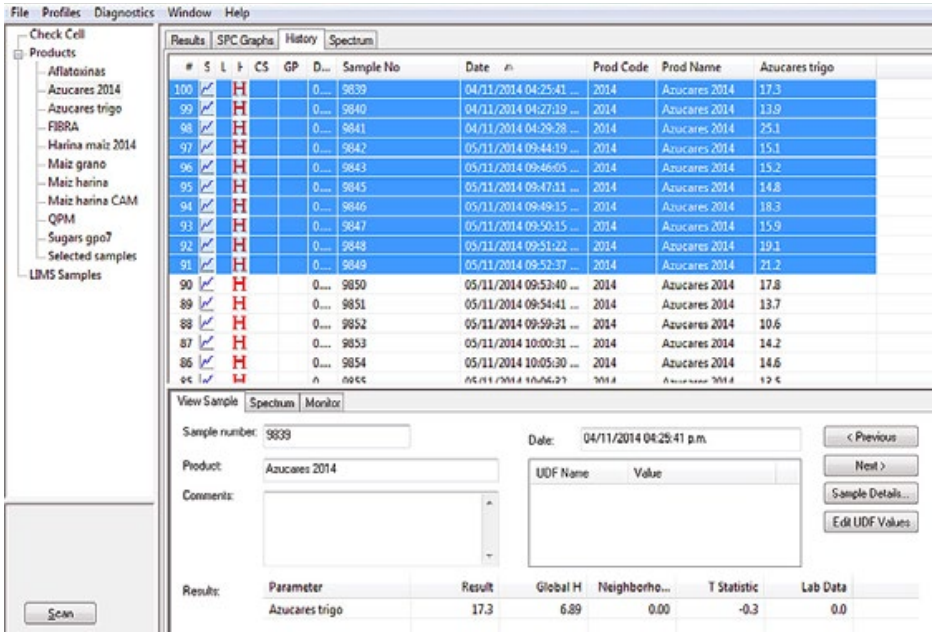
Annex Figure 22. Performance test.

Export the results

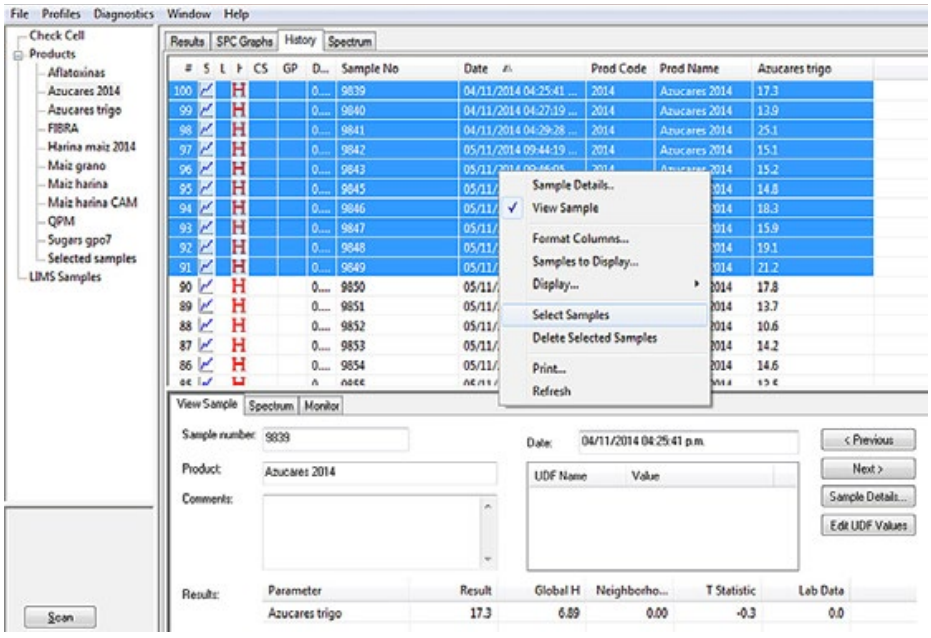
There are two options for obtaining results: the first is to export the results to an Excel, text or pdf file; the second is to obtain the spectra in ".nir file" to process it in winISI software and obtain the predicted value using a prediction equation or to use it in new calibrations.

In both cases, the first step is to select the samples from the history display using the keyboard or the mouse (Annex Figure 23). Right click on the selected samples to display the menu (Figure 16) and click on SELECT SAMPLES.

In the left menu, right click on SELECTED SAMPLES and move the cursor to the EXPORT option (Annex Figure 24).



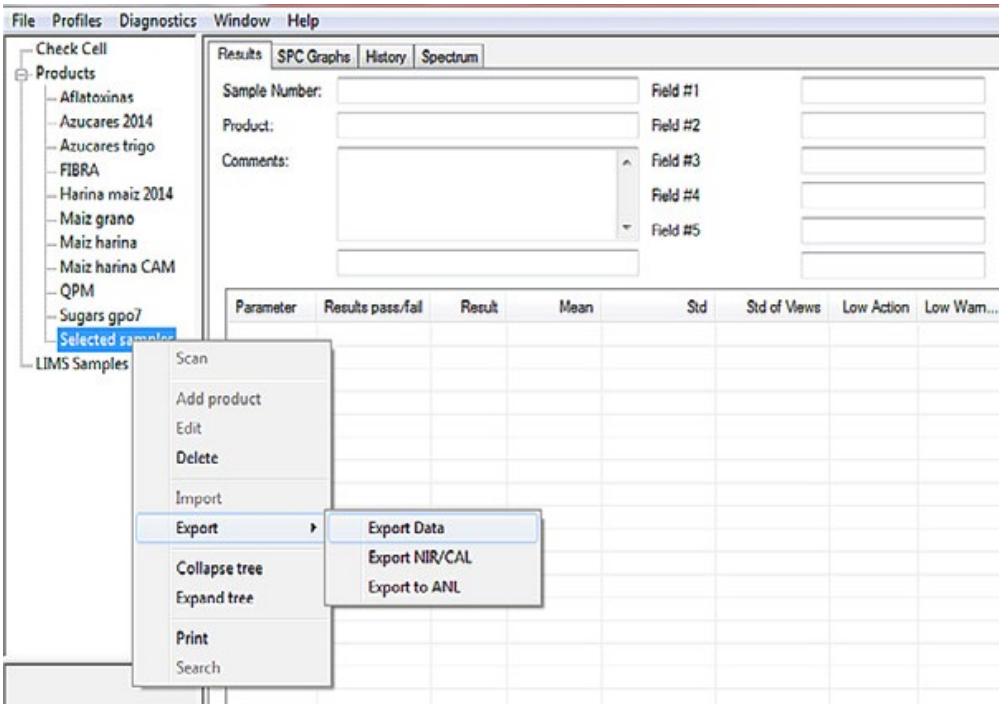
Annex Figure 23. Sample selection.



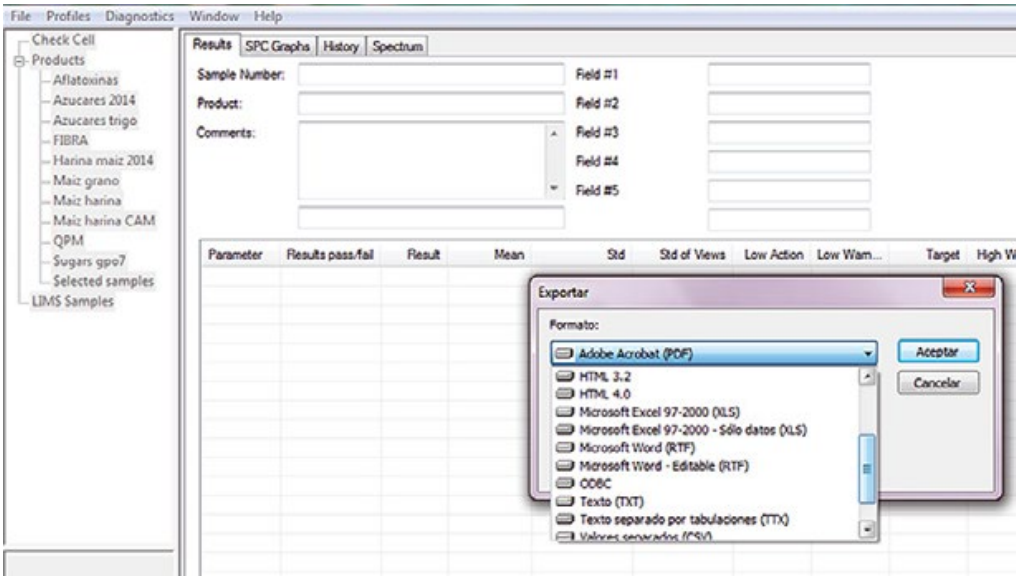
Annex Figure 24. Selecting samples.

To export results in an Excel, text or pdf file: Select the option EXPORT DATA (Annex Figure 25) and select the file format (Annex Figure 26). The file opens automatically and can be saved in any device.

To export results as spectra (NIR): Select the option EXPORT NIR/CAL (Annex Figure 25). Click on the "three points" button (Annex Figure 27), type the file name and choose the file format. It is very important to also select



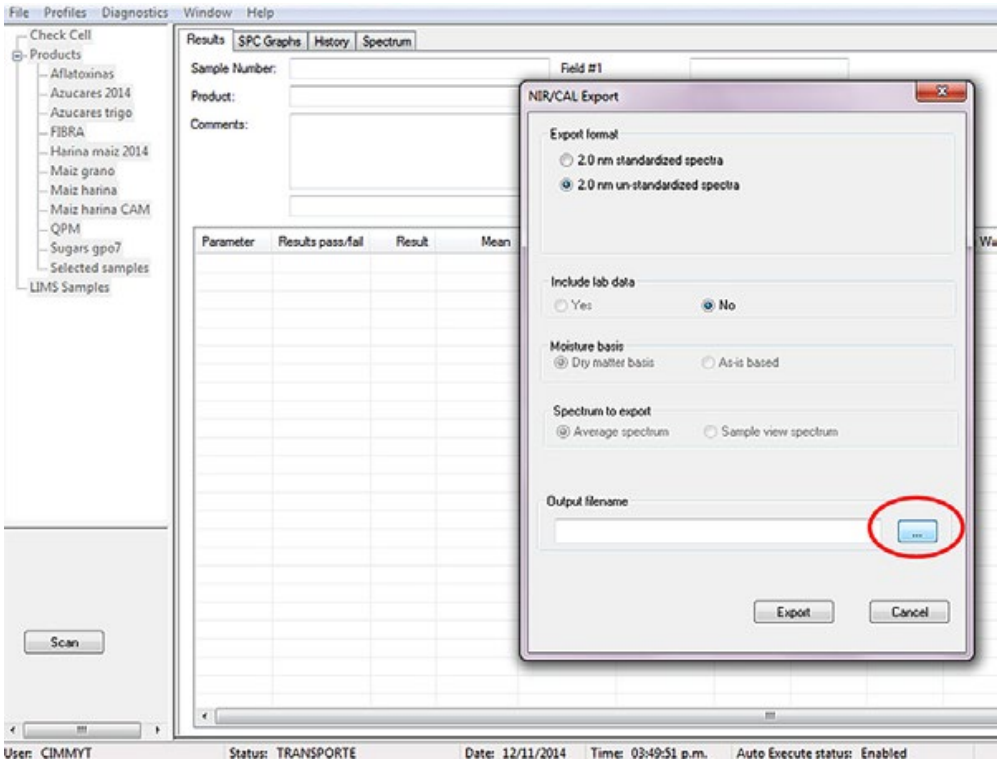
Annex Figure 25. Exporting data.



Annex Figure 26. Selecting the file format.

the option 2.0 un-standardized, which allows using the spectra in any software to process the spectra.

The process for developing equations in the WinISI software is described in the user's guide.



Annex Figure 27. Exporting spectra.

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